CHAPTER 4

RESULTS
As has already been narrated in the foregoing chapters, the objective of the present study is to investigate the role played by muscarinic and prostaglandin receptors on the release of acetylcholine from rat phrenic nerve terminals and from Auerbach's plexus of guinea-pig ileum.

The experimental findings of the study are being presented in the succeeding pages under the following broad headings:

1) Basic studies.
2) Applied studies.

4.1 BASIC STUDIES:

The results of the basic studies on the role of muscarinic receptors and prostaglandin receptors on the release of acetylcholine from motor nerve terminals and from Auerbach's plexus of guinea-pig ileum are being mentioned separately in the following pages.

4.1.1 ON THE RELEASE OF ACETYLCOLINE FROM RAT PHRENIC NERVE TERMINALS

4.1.1.1 Studies with oxotremorine:

Oxotremorine, the tremorogenic and muscarinic agent, blocks the neuromuscular transmission at the rat phrenic nerve-diaphragm junction (Ganguly and Chaudhuri, 1970), and a presynaptic action of oxotremorine at this site has been suggested (Ganguly and Chaudhuri, 1970; Ganguly, 1976). In the present study the effect of oxotremorine on the resting as well as evoked release
of acetylcholine from rat phrenic nerve terminal has been investigated. In addition, the effects of oxotremorine on the pharmacologically denervated diaphragm were investigated to determine a cause - effect relationship.

4.1.1.1.1 Effect on pharmacologically denervated rat diaphragm

Oxotremorine (10 μM) impaired twitch responses of the rat diaphragm to both 'direct' and 'indirect' stimulation (Figure 1). The effects on 'direct' twitch responses were prevented by pre-treating the preparation with hemicholinium-3, indicating that they arise from an action of oxotremorine on the nerve terminals that had been exited by current spread (Figure 1). It is concluded that oxotremorine affects transmission without having any direct action on the muscle.

4.1.1.1.2 Effect on the amount of acetylcholine released from rat phrenic nerve:

In the absence of oxotremorine (control) the total amount of acetylcholine released in the bathing fluid due to supramaximal stimulation of the phrenic nerve at a frequency of 1 Hz (0.2 ms duration) for 15 min was found to be 4.05 ± 0.73 ng and 3.4 ± 0.48 ng (mean ± s.e.) in two separate sets of experiments (Tables 1 & 2). Preincubation with a fasciculatory (2.5 μM) or a paralytic dose (10 μM) of oxotremorine, as determined by Ganguly and Chaudhuri (1970), for 15 min in the bathing fluid was followed by a sharp increase in acetylcholine release from the phrenic nerve. The quantities of acetylcholine released in presence of 2.5 and
Figure 1  Twitch responses of rat isolated diaphragm to indirect (I, left-hand traces) and direct (D, right-hand traces) stimulation. Initially oxotremorine (Oxo-T) reduced responses of both indirect and direct stimulation (a and b). After addition of hemicholinium-3 (HC-3), which blocked indirect stimulation (c), oxotremorine had no effect on direct stimulation (d). Traces (e) and (f) show effects of oxotremorine obtained 90 min after washing out hemicholinium.
Figure 2

Histogram showing dose-dependent increase in evoked release of acetylcholine from phrenic nerve terminals caused by oxotremorine. All values are normalised to the mean control acetylcholine release during 15 min periods of electrical stimulation ($N = 4.05$ ng). Values represent means of at least 6 experiments ± s.e.

* $p < 0.05$. 
Figure 3 Summary of effects of oxotremorine (2.5 and 10 μM) on the electrically evoked release of acetylcholine into the bathing fluid of rat diaphragm. The values indicate the total amount of acetylcholine released in a 4 ml bath in terms of acetylcholine chloride. Each experiment had its individual control. Vertical bars indicate mean ± s.e. *P < 0.001.
Figure 4 Histogram illustrating: a, increase in acetylcholine release from rat phrenic nerve terminals caused by 10 μM oxotremorine; b, c, d and e, dose-dependent inhibition of a by dopamine; f, reversal of e in presence of pimozide; g, influence of pimozide on a. All values are normalised to the mean control acetylcholine release during 15 min periods of electrical stimulation (N = 4.05 ng). Values represent means of at least 6 experiments ± s.e. * denotes significant increase from N (P < 0.05); † denotes significant reduction from a (P < 0.05); ‡ denotes significant increase from e (P < 0.05).
Figure 5 Histogram showing: a, increase in acetylcholine release from rat phrenic nerve terminals caused by 10 μM oxotremorine; b, c and d, dose-dependent inhibition of a by apomorphine; e, reversal of d in presence of pimozide; f, influence of pimozide on a. All values are normalised to the mean control acetylcholine release during 15 min periods of electrical stimulation (N = 4.05 ng). Values represent means of at least 6 experiments ± s.e. *denotes significant increase from N (P < 0.05); *denotes significant reduction from a (P < 0.05); *denotes significant increase from d (P < 0.05).
Figure 6  Histogram showing dose-dependent increase in evoked release of acetylcholine from phrenic nerve terminals caused by muscarine. All values are normalised to the mean control acetylcholine release during 15 min periods of electrical stimulation (N = 4.05 ng). Values represent means of at least 6 experiments ± s.e. *P < 0.05.
Figure 7  Histogram illustrating: a, increase in acetylcholine release from rat phrenic nerve terminals caused by 40 μM muscarine; b, c, and d, dose-dependent inhibition of a by dopamine; e, reversal of d in presence of pimozide; f, influence of pimozide on a. All values are normalised to the mean control acetylcholine release during 15 min periods of electrical stimulation (N = 4.05 ng). Values represent means of at least six experiments ± s.e. * denotes significant increase from N(P < 0.05); * denotes significant reduction from a (P < 0.05); * denotes significant increase from d (P < 0.05).
Figure 8

Histogram showing: a, increase in acetylcholine release from rat phrenic terminals caused by 40 μM muscarine; b, c and d, dose-dependent inhibition of a by apomorphine; e, reversal of d in presence of pimozide; f, influence of pimozide on a. All values are normalised to the mean control acetylcholine release during 15 min periods of electrical stimulation (N = 4.05 ng). Values represent means of at least six experiments ± s.e. * denotes significant increase from N (P < 0.05); * denotes significant reduction from a (P < 0.05); # denotes significant increase from d (P < 0.05).
Figure 9 Histogram illustrating: a, b and c, dose-dependent increase of evoked release of acetylcholine from rat phrenic nerve terminals by prostaglandin E₂; d, influence of atropine on c; e, influence of hexamethonium on c; f, reduction of c in presence of indomethacin; g, inhibition of c in presence of 7-oxo-13-prostynoic acid. All values are normalised to the mean control release of acetylcholine during 15 min periods of electrical stimulation (N = 4.05 ng). Values represent means of at least six experiments ± s.e.* denotes significant increase from N (P < 0.05); * denotes significant reduction from c (P < 0.05).
Figure 10: Histogram showing a, b, c, d, and e, influence of increasing concentrations of oxotremorine on evoked acetylcholine release from Auerbach's plexus of guinea-pig ileum; f, effect of atropine on control release; g influence of atropine on b. All values are expressed in percentage change taking normal release during 15 min periods of transmural stimulation (N = 1.68 ng/mg) as 100%. Values represent means of at least six experiments ± s.e. *denotes significant change from N; #denotes significant increase from b.
Figure 11 Histogram illustrating: b, c and d, dose-dependent inhibition of oxotremorine-induced increase in evoked acetylcholine release from Auerbach's plexus of guinea-pig ileum by dopamine; e, reversal of d in presence of pimozide; f, influence of pimozide on oxotremorine-induced release. All values are expressed in percentage change taking the release caused by 0.125 μM oxotremorine (C = 2.02 ng/mg) as 100% (a). Values represent means of at least six experiments ± s.e. * denotes significant reduction from a (P < 0.05); * denotes significant increase from d (P < 0.05).
Figure 12: Histogram showing influence of increasing concentrations of muscarine on evoked acetylcholine release from Auerbach's plexus of guinea-pig ileum. All values are expressed in percentage change taking normal release during 15 min periods of transmural stimulation (N = 1.66 ng/mg) as 100%. Values represent means of at least six experiments ± s.e. * denotes significant change from N; ** denotes significant increase from b; *** denotes significant increase from c.
Figure 13

Histogram illustrating: a, b, and c, dose-dependent increase in evoked release of acetylcholine from Auerbach's plexus of guinea-pig ileum by prostaglandin E₂; d, influence of indomethacin on normal release; e, influence of 7-oxa-13-prostanoic acid on normal release. All values are expressed in percentage change taking control release during 15 min periods of transmural stimulation (C = 1.63 ng/mg) as 100%. Values represent means of at least six experiments ± s.e. * denotes significant increase from C; ^ denotes significant reduction from C.
Figure 14 Histogram showing: a, reduction of prostaglandin E$_2$-induced (C) increase in evoked release of acetylcholine from Auerbach's plexus of guinea-pig ileum in presence of indomethacin; b, inhibition of C in presence of 7-oxa-13-prostynoic acid; c, influence of atropine on C. All values are expressed in percentage change taking release in presence of 24 nM prostaglandin E$_2$ during 15 min periods of transmural stimulation (C = 1.62 ng/mg) as 100%. Values represent means of at least six experiments $\pm$ s.e. * denotes significant reduction from C.
Figure 15 Histogram illustrating concentration-dependent antagonistic effects of cycrimine, benztropine and biperiden over the paralytic effects of nicotine and oxotremorine in indirectly stimulated isolated rat diaphragm preparation. T = s.e. of six experiments.
Figure 16  Structure of C$_{10}$Dichol.
Figure 17 Graph illustrating inhibition (about 50%) by C\textsubscript{10} Dichol (2 mg/kg i.p. 2 h prior) of tremor in mice induced by oxotremorine (0.25 mg/kg i.p.; ○), nicotine (0.43 mg/kg i.p.; Δ) and physostigmine (0.75 mg/kg i.p.; □). Continuous lines indicate control and broken lines after C\textsubscript{10} Dichol pretreatment. Values represent mean of 6 experiments (s.e. < 0.005 at all points).
Figure 18 Histogram illustrating the dose-dependent antagonism by C₁₀Dichol of the neuromuscular block induced by nicotine and oxotremorine on indirect twitch responses of rat diaphragm. Values represent mean of 6 experiments (T = s.e.).
Figure 19 Histogram showing the inhibition of (+) - tubocurarine (d - tc) block on indirectly elicited responses of rat diaphragm by preincubation with physostigmine and dose-dependent antagonism of this effect of physostigmine in presence of C_{10} Dichol. Values represent mean of 6 experiments (T = s.e.).
Figure 20 Rat blood pressure and heart rate: showing failure of C_{10}Dichol (C_{10}D) to inhibit the bradycardiac and vasodepressor effects of oxotremorine (Oxo) and acetylcholine (ACH).
10 μM oxotremorine were 135.14 ± 20.16 ng and 236.5 ± 33.12 ng respectively (Tables 1 & 2: Figure 2). In presence of 5 μM oxotremorine the amount of acetylcholine released was 208 ± 20.7 ng (Figure 2). In experiments with oxotremorine, the standard acetylcholine solutions used for bioassay purpose invariably contained an equivalent amount of oxotremorine, to nullify its direct influence on leech muscle, if any. This precaution was taken in spite of the fact that oxotremorine failed to influence the responses of the leech dorsal muscle to exogenous acetylcholine in separate experiments. The control release from the same diaphragm was invariably estimated either before or after (cross-over) treatment with oxotremorine. A summary of all experiments with 2.5 and 10 μM oxotremorine is shown in Figure 3. The results are expressed in terms of the weight of acetylcholine chloride.
Table 1

Effect of oxotremorine (2.5 μM) on the release of acetylcholine into the bathing fluid of rat diaphragm in response to supramaximal electrical stimulation of the phrenic nerve at 1 Hz (0.2 ms duration) for 15 min. The values indicate the total amount of acetylcholine released in a 4 ml bath in terms of acetylcholine chloride.

<table>
<thead>
<tr>
<th>No.</th>
<th>Amount of acetylcholine released in ng</th>
<th>Control</th>
<th>in presence of 2.5 μM Oxo-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td>4</td>
<td>120</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>4</td>
<td>140</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>2.4</td>
<td>100</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>3.2</td>
<td>128</td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td>7.8</td>
<td>200</td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>4.05</td>
<td>135.14</td>
</tr>
<tr>
<td>S.D.</td>
<td></td>
<td>1.94</td>
<td>53.25</td>
</tr>
<tr>
<td>S.E.</td>
<td></td>
<td>0.73</td>
<td>20.16</td>
</tr>
</tbody>
</table>
Table 2

Effect of oxotremorine (10 μM) on the release of acetylcholine into the bathing fluid of rat diaphragm in response to supramaximal electrical stimulation of the phrenic nerve at 1 Hz (0.2 ms duration) for 15 min. The values indicate the total amount of acetylcholine released in a 4 ml bath in terms of acetylcholine chloride.

<table>
<thead>
<tr>
<th>No.</th>
<th>Amount of acetylcholine released in ng</th>
<th>Control</th>
<th>In presence of 10 μM Oxo-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2.4</td>
<td></td>
<td>120</td>
</tr>
<tr>
<td>2.</td>
<td>2.4</td>
<td></td>
<td>168</td>
</tr>
<tr>
<td>3.</td>
<td>3</td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>4.</td>
<td>2.6</td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>5.</td>
<td>4</td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>6.</td>
<td>6.4</td>
<td></td>
<td>400</td>
</tr>
<tr>
<td>7.</td>
<td>4</td>
<td></td>
<td>320</td>
</tr>
<tr>
<td>Mean</td>
<td>3.4</td>
<td></td>
<td>236.5</td>
</tr>
<tr>
<td>S.D.</td>
<td>1.37</td>
<td></td>
<td>93.67</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.48</td>
<td></td>
<td>33.12</td>
</tr>
</tbody>
</table>
The amount of spontaneously released acetylcholine, in the absence of oxotremorine, could not be measured since the responses of these samples were below the linear sensitivity range of the bioassay preparation. As with nerve stimulation oxotremorine (2.5 and 10 μM) produced a large increase in the amount of acetylcholine released during the resting period. The amounts released were 12.0 ± 1.45 and 29.3 ± 6.02 (Table 3) in the presence of 2.5 and 10 μM oxotremorine respectively (control was not measurable).

**Table 3**

Effect of oxotremorine on spontaneous release of acetylcholine into the bathing fluid of rat diaphragm in 15 min. The values indicate the total amount of acetylcholine released in a 4 ml bath in terms of acetylcholine chloride.

<table>
<thead>
<tr>
<th>No.</th>
<th>Amount of acetylcholine released in ng</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1.</td>
<td>Not measurable (necessarily &lt;1.25)</td>
</tr>
<tr>
<td>2.</td>
<td>- do -</td>
</tr>
<tr>
<td>3.</td>
<td>- do -</td>
</tr>
<tr>
<td>4.</td>
<td>- do -</td>
</tr>
<tr>
<td>5.</td>
<td>- do -</td>
</tr>
<tr>
<td>6.</td>
<td>- do -</td>
</tr>
<tr>
<td>Mean</td>
<td>-</td>
</tr>
<tr>
<td>S.D.</td>
<td>-</td>
</tr>
<tr>
<td>S.E.</td>
<td>-</td>
</tr>
</tbody>
</table>
4.1.1.3 Effect of atropine on control and oxotremorine induced acetylcholine release

The increase in the release of acetylcholine in presence of oxotremorine (10 μM) from the rat phrenic nerve terminals was completely inhibited in presence of 0.5 μM atropine (Table 4). Singular incubation of atropine in control experiments inhibited the electrically evoked release of acetylcholine to a level below the sensitivity range of the bioassay preparation (Table 4). The acetylcholine releasing effect of oxotremorine from rat phrenic nerve terminals and its complete prevention by atropine demonstrated presence of excitatory presynaptic muscarinic receptors on motor nerve terminals.

4.1.1.4 Influence of dopamine receptors on oxotremorine induced release of acetylcholine

In view of strong evidence for a muscarinic cholinergic–dopaminergic link in the central nervous system (Sethy and Van Woert, 1973; Westfall, 1974a; Trabucchi, Cheney, Racagni and Costa, 1975a), the influence of dopamine, apomorphine (dopamine receptor agonist), and pimozide, the dopamine antagonist, on acetylcholine releasing effect of oxotremorine from phrenic nerve terminals was investigated.

Dopamine, at concentrations (upto 0.05 μM) which failed to alter the normal evoked release of acetylcholine by itself (Table 4), inhibited the profound increase in evoked acetylcholine release, caused by oxotremorine (10 μM) as a function of its concentration (Figure 4). However, a higher (×100) concentration of dopamine
(5 μM) inhibited the evoked release of acetylcholine to a degree which could not be measured (Table 4). The inhibitory effect of dopamine (0.05 μM) on oxotremorine induced release of acetylcholine was nullified in presence of pimozide (0.25 μM), the dopamine receptor antagonist (Figure 4). Unlike with atropine, both the control and oxotremorine-induced release of acetylcholine remained unaltered in presence of pimozide (Table 4; Figure 4).

Table 4

Effect of different drugs on acetylcholine release. Figures show acetylcholine released, in the form of acetylcholine chloride, into a 4 ml bath from rat phrenic nerve terminals in response to supramaximal stimulation (0.2 ms) at 1 Hz for 15 min in different experimental conditions. Values represent mean of at least six experiments ± s.e. Concentrations of drugs are expressed in terms of molarity of the salts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetylcholine released (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.05 ± 0.73</td>
</tr>
<tr>
<td>Atropine sulphate 0.5 μM</td>
<td>Not measurable (necessarily &lt;1.25)</td>
</tr>
<tr>
<td>Atropine sulphate 0.5 μM + oxotremorine 10 μM</td>
<td>- do -</td>
</tr>
<tr>
<td>Dopamine hydrochloride 5 μM</td>
<td>- do -</td>
</tr>
<tr>
<td>Dopamine hydrochloride 0.05 μM</td>
<td>4.0 ± 1.1</td>
</tr>
<tr>
<td>Pimozide 0.25 μM</td>
<td>3.2 ± 0.42</td>
</tr>
</tbody>
</table>
Like dopamine, apomorphine (1.5, 7.5 and 30 nM) also inhibited the oxotremorine (10 μM) induced increase in evoked release acetylcholine from rat phrenic nerve terminals as a function of its concentration (Figure 5). The inhibitory effect of apomorphine (30 nM) on oxotremorine (10 μM) induced release of acetylcholine was nullified in presence of 0.25 μM pimozide (Figure 5).

The antagonism of the presynaptic muscarinic effect of oxotremorine on acetylcholine release by dopamine and apomorphine, and reversal of this effect of the dopaminergic agents by pimozide suggest that dopaminergic sites which exert an inhibitory influence on the excitatory presynaptic muscarinic receptors exist on motor nerve terminals.

4.1.1.2 Effect of muscarine on acetylcholine release from rat phrenic nerve

Muscarine chloride, another muscarinic agonist, was investigated for its acetylcholine releasing effect from rat phrenic nerve ending. Like oxotremorine, muscarine also enhanced the evoked release of acetylcholine from phrenic nerve terminals in a dose-dependent manner (Figure 6). The amount of acetylcholine released in presence of 10, 20 and 40 μM of muscarine chloride were 20.2 ± 2.4 ng, 33.6 ± 2.9 ng and 51.2 ± 5.2 ng respectively.

Preincubation with 0.5 μM atropine sulphate inhibited the increase in evoked release of acetylcholine induced by muscarine (40 μM) to a level below the sensitivity range of the bio-assay preparation.
4.1.1.2.1 Influence of dopamine receptors on muscarine-induced acetylcholine release

Dopamine (0.02, 0.03 and 0.05 μM) inhibited the increase in evoked release of acetylcholine caused by muscarine (40 μM) as a function of its concentration (Figure 7). The inhibitory effect of dopamine (0.05 μM) was reversed by pimozide (0.25 μM), the dopamine receptor antagonist (Figure 7). The muscarine (40 μM) induced release of acetylcholine, however, remained unaltered in presence of 0.25 μM of pimozide (Figure 7).

Like dopamine, apomorphine (0.003 to 0.012 μM) also antagonised in a dose-dependent manner the increase in evoked release of acetylcholine from rat phrenic nerve terminals caused by 40 μM of muscarine chloride (Figure 8). The antagonistic effect of apomorphine (0.012 μM) was nullified in presence of pimozide (Figure 8).

The antagonism of the muscarinic presynaptic effect of muscarine by dopamine and apomorphine and the reversal of this effect of dopamine agonists by pimozide further confirm that dopaminoceptive sites, which exert an inhibitory influence on the excitatory muscarinic receptors, are present on motor nerve terminals.

4.1.1.3 Effect of prostaglandin E₂ on acetylcholine release from phrenic nerve terminals

Evidence in the literature suggest that prostaglandin releases acetylcholine from myenteric plexus of guinea-pig ileum (Yagasaki and Takai, 1973; Hall, O'Neill and Sheehan, 1975; Kadlec, Masek and Seffern, 1978). In the present study the influence of
prostaglandin E\(_2\) on acetylcholine release from rat phrenic nerve terminal was investigated.

Prostaglandin E\(_2\) produced a dose-dependent increase of evoked release of acetylcholine from rat phrenic nerve terminals (Figure 9). The amount of acetylcholine released in presence of 0.0014, 0.0028 and 0.014 \(\mu\)M prostaglandin E\(_2\) were 12.6 \(\pm\) 3.95 ng, 28.3 \(\pm\) 4.6 ng and 39.4 \(\pm\) ng respectively.

Preincubation with atropine (0.5 \(\mu\)M) and hexamethonium (56 \(\mu\)M) failed to significantly block the increase in acetylcholine release caused by 0.014 \(\mu\)M prostaglandin E\(_2\) (Figure 9). However, the prostaglandin receptor blocker 7-oxa-13-prostynoic acid (0.032 \(\mu\)M), and indomethacin (2.6 \(\mu\)M) inhibited the prostaglandin E\(_2\) (0.014 \(\mu\)M) induced increase in evoked release of acetylcholine (Figure 9). Singular administration of either 7-oxa-13-prostynoic acid (0.032 \(\mu\)M) or indomethacin (2.6 \(\mu\)M) did not alter the control evoked acetylcholine release.

4.1.2 ON THE RELEASE OF ACETYLCHOLINE FROM AUERBACH'S PLEXUS OF GUINEA-PIG ILEUM

The effect of various agents on spontaneous as well as electrically (field stimulation) evoked release of acetylcholine from the Auerbach's plexus of guinea-pig ileum was investigated according to the method described earlier in detail (see Materials and methods).
4.1.2.1 Effect of oxotremorine

The control (in absence of any agent) release of acetylcholine into the bathing fluid in response to supramaximal field stimulation of the Auerbach's plexus at a frequency of 1 Hz (1 ms duration) for 15 min was found to be 1.68 ± 0.46 ng/mg (mean ± s.e.).

Preincubation with lower concentrations (0.025 μM, 0.125 μM and 0.25 μM) of oxotremorine for 15 min in the bathing fluid was followed by an increase in evoked acetylcholine release from the Auerbach's plexus (Figure 10). The percentage increase in the amount of released acetylcholine in presence of 0.025 μM, 0.125 μM and 0.25 μM oxotremorine were 14 ± 67.3, 140.4 ± 67.8 and 94.4 ± 69.4 respectively. However, preincubation with higher concentrations of oxotremorine (5 and 10 μM) for 15 min in the bathing fluid was followed by a dose-dependent inhibition of evoked release of acetylcholine from Auerbach's plexus. The percentage inhibition of acetylcholine release caused by oxotremorine at these concentrations were 28.2 ± 4.2 and 70.24 ± 7.9 respectively. The results are summarised in Figure 10.

The amount of spontaneously released acetylcholine from Auerbach's plexus in the absence of oxotremorine was 0.72 ± 0.18 ng/mg/15 min (mean ± s.e.). Oxotremorine (0.025 to 10 μM) did not produce any significant change in the amount of spontaneously released acetylcholine.
4.1.2.1.1 Influence of atropine on the effect of oxotremorine

The increase in evoked release of acetylcholine from Auerbach's plexus caused by oxotremorine (0.125 μM) was facilitated in presence of 1 μM of atropine (Figure 10). Singular incubation of atropine (1 μM) in control experiments also increased the electrically evoked release of acetylcholine from Auerbach's plexus (Figure 10).

4.1.2.1.2 Effect of dopamine receptors on oxotremorine-induced acetylcholine release from Auerbach's plexus

In view of strong evidence for presynaptic modulation of acetylcholine release by dopamine in the peripheral and central nervous systems (for references, see Vizi, Rónai, Harsing and Knoll, 1977b), the influence of dopamine and its receptor antagonist, pimozide on the acetylcholine releasing effect of oxotremorine from Auerbach's plexus was examined.

Dopamine (0.0025, 0.005 and 0.01 μM) inhibited the increase in evoked release of acetylcholine from Auerbach's plexus induced by oxotremorine (0.125 μM) as a function of its concentration (Figure 11). The inhibitory effect of dopamine on oxotremorine-induced increase in release of acetylcholine was nullified in presence of 0.25 μM pimozide (Figure 11). The oxotremorine-induced release of acetylcholine from Auerbach's plexus remained unaltered in presence of 0.25 μM pimozide (Figure 11).

Singular administration of the highest concentration (0.01 μM) of dopamine used in the present study and pimozide (0.25 μM) failed to significantly alter the normal evoked release of acetylcholine from Auerbach's plexus; the percentage increase in acetylcholine
release caused by dopamine (0.01 μM) and pimozide (0.25 μM) were 5.3 ± 7.4 and 0.5 ± 10.2 respectively.

4.1.2.2 Effect of muscarine

Preincubation with lower concentrations (1.1 and 2.2 μM) of muscarine for 15 min in the bathing fluid was followed by a concentration dependent increase in evoked release of acetylcholine from the Auerbach's plexus (Figure 12). The percentage increase in the amount of released acetylcholine in presence of 1.1 and 2.2 μM muscarine were 75.6 ± 20.8 and 247.6 ± 24.8 respectively. A higher concentration (10 μM) of muscarine inhibited the evoked release of acetylcholine from Auerbach's plexus (Figure 12); it caused a percentage inhibition of 33.7 ± 10.2.

The maximum increase in the evoked release of acetylcholine caused by muscarine (2.2 μM) was further increased in presence of 1 μM atropine (Figure 12). Furthermore, the inhibition of acetylcholine release induced by 10 μM muscarine was changed into facilitation of release in presence of 1 μM atropine (Figure 12).

4.1.2.3 Effect of prostaglandin

Prostaglandin E₂ (6, 12 and 24 nM) increased the evoked release of acetylcholine from the Auerbach's plexus in a concentration dependent manner (Figure 13). The percentage increase of evoked release of acetylcholine caused by 6, 12 and 24 nM prostaglandin E₂ were 12.2 ± 10.3, 64.9 ± 11.9 and 271.3 ± 34.1 respectively.
The maximum increase in acetylcholine release induced by prostaglandin E$_2$ (24 nM) was significantly inhibited in presence of either 1 µM indomethacin or 0.032 µM 7-oxa-13-prostynoic acid (Figure 14). Preincubation with atropine (1 µM) failed to significantly change the increase in evoked acetylcholine release caused by 24 nM prostaglandin E$_2$ (Figure 14).

Singular incubation for 15 min with either indomethacin (1 µM) or 7-oxa-13-prostynoic acid (0.032 µM) significantly inhibited the control evoked release of acetylcholine from Auerbach's plexus (Figure 13).

While investigating the influence of various agents on the release of acetylcholine from Auerbach's plexus of guinea-pig ileum, the standard acetylcholine solutions used for bioassay purpose invariably contained an equivalent amount of the respective agent/agents, to nullify its/their direct influence on leech muscle, if any.

The control release from the same tissue was invariably estimated either before or after (cross-over) treatment with the agent/agents.

4.2 APPLIED STUDIES

4.2.1 Interaction between some cholinolytic anti-Parkinson drugs with nicotine and oxotremorine on rat diaphragm

Nicotine and oxotremorine, the two tremorgenic agents commonly employed to induce Parkinson-like symptoms in experimental animals for the purpose of evaluation of anti-Parkinson efficacy of new agents, affect the skeletal muscle and its neurotransmission
(Rummel and Schulz, 1954a&b; Ganguly and Chaudhuri, 1970; Ganguly, 1976). With the view to examine the role played by the neuromuscular effect of nicotine and oxotremorine in the production of Parkinson-like features in experimental animals, the influence of three centrally acting cholinolytic anti-Parkinson agents, namely cycrimine, benztropine and biperiden on the neuromuscular blocking effects of the two tremorogenic agents was studied.

For this purpose, the submaximal paralytic doses of oxotremorine (0.01 mM) and nicotine (0.06 mM) were used throughout. The anti-Parkinson agents were preincubated for 5 min before administration of the tremorogenic agents in the bathing fluid.

Preincubation of the anti-Parkinson agents reduced the neuromuscular paralytic effects of nicotine and oxotremorine in a concentration dependent manner (Figure 15). The doses of the anti-Parkinson drugs used ranged between 0.15 to 0.6 μM for cycrimine, 0.47 to 1.88 μM for benztropine and 0.05 to 0.4 μM for biperiden (Figure 15). No further increase in the antagonism occurred by increasing the doses of anti-Parkinson drugs and complete antagonism was not observed in any of the experiments. At the dose-range employed, the anti-Parkinson agents did not affect the responses of indirectly elicited twitch responses of the rat diaphragm preparation.

4.2.2 Anti-tremor action of C₁₀Dichol, a peripheral acetylcholine synthesis inhibitor

In view of the finding that a cholinergic dominance at extra- and intra-fusal neuromuscular sites is involved in experimental parkinsonogenesis (Cahen and Lynes, 1951; Ganguly and Chaudhuri,
1970; Chaudhuri and Ganguly; 1974; Ganguly, 1976; Fackler, Ross, Cleveland and Haase, 1977; Ganguly, Nath, Ross and Vedasiromoni, 1978) and the observation that skeletomotor site plays a role in the anti-Parkinson action of agents in current therapeutic use (Onuaguluchi and Lewis, 1963; Onuaguluchi, 1964; Salako, 1970; Vedasiromoni and Ganguly, 1976), developing a peripheral anti-Parkinson drug acting at skeletomotor site appeared plausible. The candidature of C\textsubscript{10}Dichol, decamethylene-bis-(hydroxyethyl)-dimethyl-ammonium bromide, as a possible anti-Parkinson agent looked to be promising as it does not permeate the blood brain barrier (Figure 16), has a considerably higher margin of safety as compared to hemicholinium-3, the well known inhibitor of acetylcholine synthesis, and impairs neuromuscular transmission (Barlow and Zoller, 1962; Bowman and Hemsworth, 1965b; Hemsworth, 1971).

In this phase of the study investigation of the interaction between C\textsubscript{10}Dichol and some tremorogenic agents at skeletomotor junction and screening of C\textsubscript{10}Dichol's anti-Parkinson potentiality have been made.

4.2.2.1 Effect of C\textsubscript{10}Dichol on tremor

Tremor induced by oxotremorine (250 μg/kg i.p.), nicotine (430 μg/kg i.p.), and physostigmine (750 μg/kg i.p.) were preferentially inhibited by pretreatment (2 h) with C\textsubscript{10}Dichol at doses between 0.5 and 4 mg/kg i.p. The dose of C\textsubscript{10}Dichol affording about 50% tremor protection was found to be 2 mg/kg i.p., common to all the three tremorogens. The results are illustrated in Figure 17.

Tremor induced by harmine (10 mg/kg i.p.) and arecoline (10 mg/kg i.p.)
remained uninhibited after pretreatment (2 h) with C₁₀Dichol (0.5 to 4 mg/kg i.p.).

4.2.2.2 Skeletal myoneural studies with C₁₀Dichol

Upon prior incubation (5 min), C₁₀Dichol prevented as a function of its concentration the submaximal paralytic effect of oxotremorine (10 μM) and nicotine (0.06 mM) on indirect twitch responses of the rat phrenic nerve-diaphragm preparation (Figure 18). The neuromuscular blocking effects caused by two other tremorogens, namely harmine (0.2 mM) and arecoline (5 mM), remained unaltered by preincubation of C₁₀Dichol.

In another set of experiments, interaction of C₁₀Dichol with physostigmine was studied by examining the influence of C₁₀Dichol on the ability of physostigmine to antagonise the neuromuscular blocking effect of (+)-tubocurarine (d-tc). Prior incubation (5 min) with physostigmine (4 μM) completely reversed the paralytic effect of (+)-tubocurarine on indirect twitch responses of the diaphragm and this antagonistic effect of physostigmine was reversed dose-dependently by C₁₀Dichol (0.01 to 0.08 mM) when administered in the bathing fluid 5 min prior to physostigmine (Figure 19).

In control experiments, C₁₀Dichol up to 0.08 mM failed to inhibit the indirectly elicited twitch responses of rat diaphragm. C₁₀Dichol (0.01 to 0.08 mM) potentiated the responses of isolated frog rectus abdominis muscle to submaximal doses (5 μM) of acetylcholine and inhibited the blocking effect of (+)-tubocurarine (0.06 μM) on such responses.
4.2.2.3 Effect of C_{10}Dichol on physostigmine-induced death

Pretreatment (2 h) with C_{10}Dichol (0.5 to 2 mg/kg i.p.) protected mice to a maximum of 50% (18/35 mice) against a challenge LD_{99} dose (2 mg/kg i.p.; Nose and Kojima, 1970) of physostigmine. Increase in the dose of C_{10}Dichol above 2 mg/kg failed to afford any further protection.

4.2.2.4 Effect of C_{10}Dichol on muscarinic receptors

Vasodepressor and bradycardiac responses to oxotremorine and acetylcholine in rats remained unaltered when repeated 1 h after administration of C_{10}Dichol up to a dose of 2 mg/kg i.p. A typical record is shown in Figure 20. A transient vasodepressor effect lasting for a maximum of 10 min was observed after injection of C_{10}Dichol (Figure 20).