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PRESYNAPTIC MUSCARINIC RECEPTORS INHIBITING ACTIVE ACETYLCHOLINE RELEASE IN THE BULLFROG SYMPATHETIC GANGLION

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1 The effects of bethanechol and atropine on the release of acetylcholine (ACh) from bullfrog sympathetic preganglionic nerve terminals were examined electrophysiologically.

2 Bethanechol (1 mM) caused no depolarization of sympathetic preganglionic nerve terminals, whereas carbachol or ACh in the same concentration induced marked depolarizations of these terminals.

3 Bethanechol (10 μM) depressed the amplitude of fast excitatory postsynaptic potentials (e.p.s.ps) recorded in low Ca^{2+}-high Mg^{2+} solution, without depolarizing ganglion cells. The quantal content measured from these fast e.p.s.ps by the variance method showed a significant reduction.

4 Amplitudes of both miniature e.p.s.ps and ACh-potentials induced by iontophoresis of ACh were not affected by addition of bethanechol (10 μM).

5 The depressant effect of bethanechol (10 μM) on fast e.p.s.ps disappeared in the presence of atropine (3 μM).

6 Atropine (3 μM) increased the quantal content measured from fast e.p.s.ps recorded in low Ca^{2+}-high Mg^{2+} solution.

7 The depressant effect of bethanechol (10 μM) on fast e.p.s.ps was unaffected by α-adrenoceptor blocking agents (phenoxybenzamine (10 μM) or phentolamine (10 μM)).

8 These results suggest that presynaptic nerve terminals in bullfrog sympathetic ganglia possess a muscarinic receptor which inhibits active release of ACh.

Introduction

Some cholinergic synapses appear to possess presynaptic muscarinic receptors which inhibit acetylcholine (ACh) release by a negative feed-back mechanism. In 1956, Schaumann reported that atropine increased ACh release in guinea-pig intestine. It was suggested that the myenteric plexus in guinea-pig ileum had a muscarinic receptor which modulated ACh release by a negative feed-back mechanism (Kilbinger & Wagner, 1975; Sawynok & Jhamandas, 1977; Kilbinger, 1977; Fosbraey & Johnson, 1978; Kilbinger & Wagner, 1979; Gustafsson, Hasqvist & Lundgren 1980; Kilbinger & Wessler, 1980a,b; Fosbraey & Johnson, 1980; Kilbinger & Kruel, 1981). Furthermore, it has been suggested that similar presynaptic muscarinic receptors exist in cerebral cortex of the cat (Dudar & Szerb, 1969), in central cholinergic nerves of rat (Polak, 1967; 1971; Szerb & Somogyi, 1973; Kato, Collier, Ilson & Wright, 1975; Hadházy & Szerb, 1977; Szerb, Hadházy & Dudar, 1977; Szerb, 1977; Nordström & Bartfai, 1980), in the electric organ of *Torpedo* (Michaelson, Avissar, Kloog & Sokolovsky, 1979; Dunant & Walker, 1981) and in rat phrenic nerve-diaphragm preparations (Abbs & Joseph, 1981). On the other hand, Kato *et al.* (1975) found that the cat superior cervical ganglion did not have such a presynaptic muscarinic receptor.

In the present experiments, we analysed electrophysiologically the effects of bethanechol and atropine on ACh-release in bullfrog sympathetic ganglia. The results strongly suggest that presynaptic nerve terminals in bullfrog sympathetic ganglia possess a muscarinic receptor which inhibits active release of ACh.

Methods

The ninth or tenth lumbar sympathetic ganglia of bullfrogs (*Rana catesbeiana*) were isolated and continuously perfused with Ringer solution throughout the experiment. Experimental arrangements used for the sucrose-gap method were similar to those described previously (Koketsu & Nishi, 1968). Techniques used for intracellular recording of membrane potential of ganglion cells followed the method of

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Nishi & Koketsu (1960); only B type cells (Nishi, Soeda & Keketsu, 1965) were used. Glass microelectrodes filled with 3 M KCl with tip resistances of 20–40 MΩ and small junctional potentials were used. The ACh sensitivity of the cell membrane was measured by recording nicotinic ACh-potentials produced by iontophoretic application of ACh through a microelectrode (50–100 MΩ) filled with 2 M ACh-chloride (Koketsu, Nishi & Soeda, 1968). The composition of the Ringer solution was as follows (mM): NaCl 112, KCl 2, $CaCl_2$ 1.8 and $NaHCO_3$ 2.4. To record the spontaneous miniature e.p.s.ps, the concentration of KCl was increased to 10 mM. Fast e.p.s.ps were recorded by applying preganglionic nerve stimulation at a rate of 0.2 Hz in a low Ca^{2+}-high Mg^{2+} solution (NaCl 112, KCl 2, $CaCl_2$ 0.5 to 0.8, $MgCl_2$ 5.5 to 7.0 and $NaHCO_3$ 2.4) or in a Ringer solution containing (+)-tubocurarine (Tc). The mean quantal content of fast e.p.s.ps was calculated by the variance method (del Castillo & Katz, 1954; Blackman, Ginsborg & Ray, 1963) from mean amplitude and its standard deviation of 60 fast e.p.s.ps induced for 5 min before and during an application of a test drug. Changes in the quantal content during application of a drug were expressed by the percentage change in the value of mean quantal content. All experiments were carried out at room temperature (20–24°C). Drugs used were: ACh chloride (Wako Pure Chemical Co.), atropine sulphate (Merck Co.), bethanechol chloride (Sigma Chemical Co.), carbachol (carbamylcholine chloride) (Merck Co.), (+)-tubocurarine chloride (Sigma Chemical Co.), phenoxybenzamine hydrochloride (Tokyo Kasei Co.) and phentolamine hydrochloride (Ciba-Geigy Co.).

Results

Effects of bethanechol on the membrane potential of preganglionic nerve terminals

It has been known that preganglionic nerve terminals in sympathetic ganglia possess nicotinic cholinoceptors (Koketsu & Nishi, 1968; Ginsborg, 1971). Thus, the membrane of preganglionic nerve terminals is depolarized by the nicotinic action of ACh or carbachol (Koketsu & Nishi, 1968; Ginsborg, 1971), and such a depolarization of nerve terminals can be recorded by use of the sucrose-gap method. Figure 1 shows changes in the membrane potential of preganglionic nerve terminals of a bullfrog sympathetic ganglion in the presence of ACh, carbachol and bethanechol. As seen in this figure, the membrane of preganglionic nerve terminals is markedly depolarized by ACh and carbachol, whereas it was not depolarized at all by the action of bethanechol. These

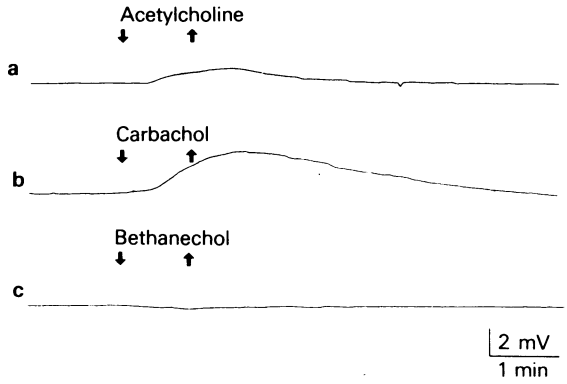

Figure 1 Effects of (a) acetylcholine (ACh), (b) carbachol and (c) bethanechol in a concentration of 1 mM on the membrane potential of preganglionic nerve terminals. These results were obtained from a single preparation by the sucrose-gap method; note depolarization induced by ACh or carbachol and its absence with bethanechol.

results indicated that the membrane of preganglionic nerve terminals was indeed not depolarized by the muscarinic action of bethanechol.

Effects of bethanechol on fast e.p.s.ps

Figure 2 shows the effect of 10 μM bethanechol on fast e.p.s.ps recorded in a low Ca^{2+}-high Mg^{2+} solution. The amplitude of fast e.p.s.ps is decreased during applications of bethanechol, as seen in Figure 2a. The mean amplitude of fast e.p.s.ps decreased to $71.4 \pm 5.2\%$ ($n = 17$) of the control value 10 min after an application of bethanechol; numbers are mean ± standard error (s.e.) and n = numbers of cells observed. The mean quantal content of fast e.p.s.ps decreased to $69.6 \pm 4.0\%$ ($n = 17$) of the control value 10 min after an application of bethanechol. In Figure 2b, the time course of changes in the mean quantal content of fast e.p.s.ps before, during and after an application of bethanechol is presented. The amplitude of fast e.p.s.ps recorded in a Ringer solution containing (+)-Tc (15–30 μM) was decreased to a comparable degree during applications of bethanechol (10 μM). Bethanechol in this concentration caused no significant changes in the resting membrane potential of the postganglionic cells in both low Ca^{2+}-high Mg^{2+} solution and Ringer solution containing (+)-Tc (Suzuki & Volle, 1978).

Effects of bethanechol on miniature e.p.s.ps and acetylcholine potentials

Figures 3a and b show the effect of 10 μM bethanechol on miniature e.p.s.ps in a high K^+ Ring-

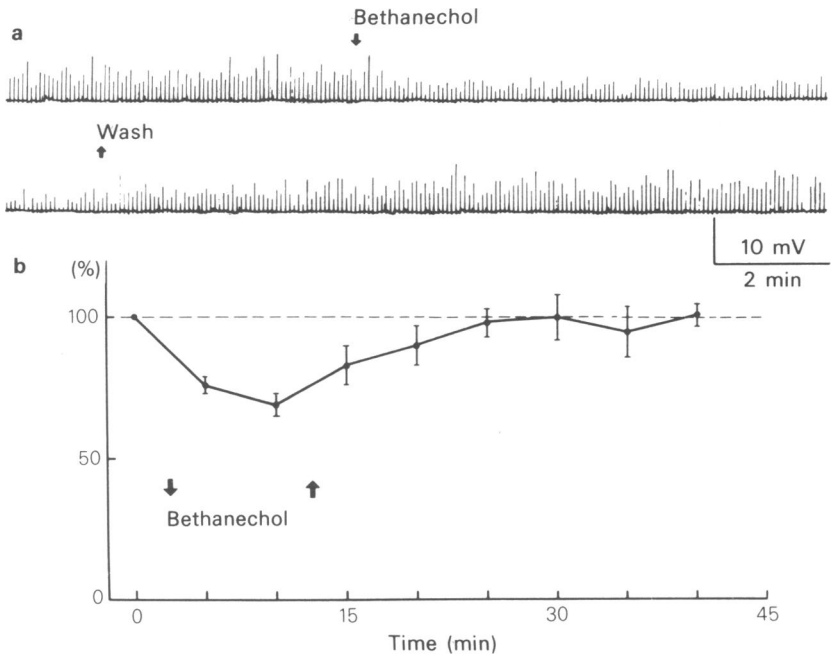

Figure 2 (a) Effect of bethanechol (10 μM) on fast e.p.s.ps in a low Ca^{2+}-high Mg^{2+} solution. Arrows indicate the period when bethanechol was present in the bathing solution. (b) Effect of bethanechol (10 μM) on the mean quantal content of fast e.p.s.ps. Ordinate scale shows the percentage change in the mean quantal content of fast e.p.s.ps; mean values are shown and vertical lines indicate s.e.mean ($n = 17$). Arrows indicate the period of application of bethanechol.

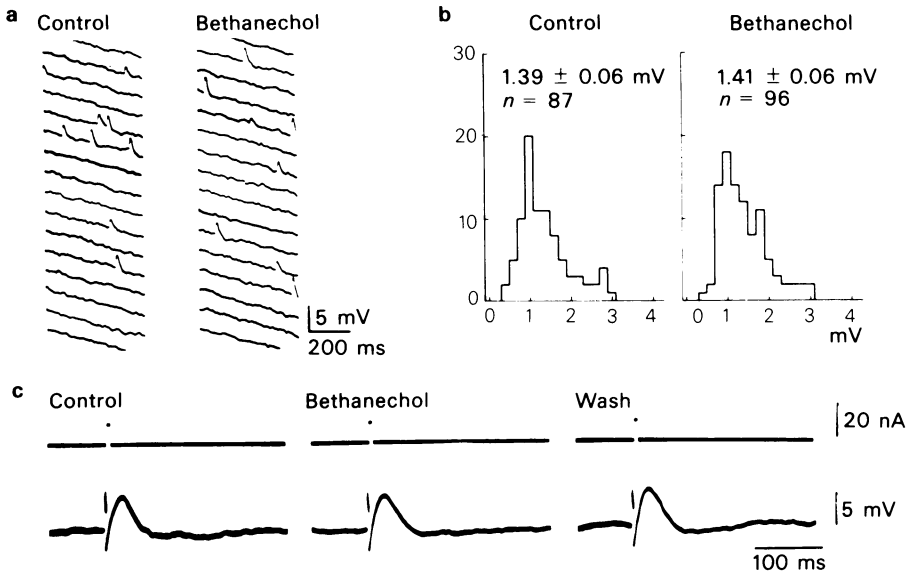

Figure 3 (a) Miniature e.p.s.ps before and during application of bethanechol (10 μM). (b) Histograms showing amplitude of miniature e.p.s.ps before (10 min) and during (10 min) application of bethanechol. Ordinates indicate numbers of miniature e.p.s.ps and abscissae the amplitude. The mean amplitude ± s.e. is shown and n is the number of miniature e.p.s.ps during 10 min. (c) Effect of bethanechol (10 μM), on the potential induced by iontophoretic application of acetylcholine (ACh) before, during and after application of bethanechol. Upper and lower traces are the currents for iontophoresis and membrane potential, respectively.

er solution. The mean amplitude of miniature e.p.s.ps shows no detectable changes in the presence of 10 μM bethanechol. Bethanechol in this concentration caused no significant changes in the frequency of miniature e.p.s.ps. Figure 3c shows the effect of 10 μM bethanechol on the potential induced by iontophoretic application of ACh to the ganglion cell in the Ringer solution. The amplitude of the ACh-potential shows no detectable change in the presence of bethanechol at this concentration.

Effects of bethanechol on fast e.p.s.ps in the presence of atropine

Figure 4 shows the effects of 10 μM bethanechol on fast e.p.s.ps in the presence of 3 μM atropine. When a ganglion was previously immersed 30 min or more in a low Ca^{2+}-high Mg^{2+} solution containing 3 μM atropine, the amplitude of these fast e.p.s.ps was not affected by addition of 10 μM bethanechol, as seen in Figure 4a. The mean quantal content of these fast e.p.s.ps does not decrease; $104.7 \pm 8.7\%$ ($n = 7$) during an application of 10 μM bethanechol.

Effects of atropine on fast e.p.s.ps miniature e.p.s.ps and acetylcholine potentials

Effects of atropine on the release of ACh from preganglionic nerve terminals were studied by recording fast e.p.s.ps in a low Ca^{2+}-high Mg^{2+} solution. Effects on miniature e.p.s.ps and ACh-potentials were studied in a high K^+ Ringer solution and normal Ringer solution, respectively. It was found in a preliminary experiment that atropine (3–30 μM) facilitated the active release of ACh while it depressed the sensitivity of nicotinic receptors by decreasing the amplitude of miniature e.p.s.ps and ACh-potentials (Yamada, 1981). When atropine in a concentration of 3 μM was used, the active release of ACh was clearly increased while the sensitivity of ACh-receptors was slightly depressed. The results obtained with 3 μM atropine are seen in Figures 5 and 6, which show the change in quantal content of fast e.p.s.ps and the changes in amplitudes of miniature e.p.s.ps and ACh-potentials, respectively. The mean amplitude of fast e.p.s.ps showed no significant changes ($103.1 \pm 3.1\%$; $n = 14$) while the mean quantal content of fast e.p.s.ps increased to

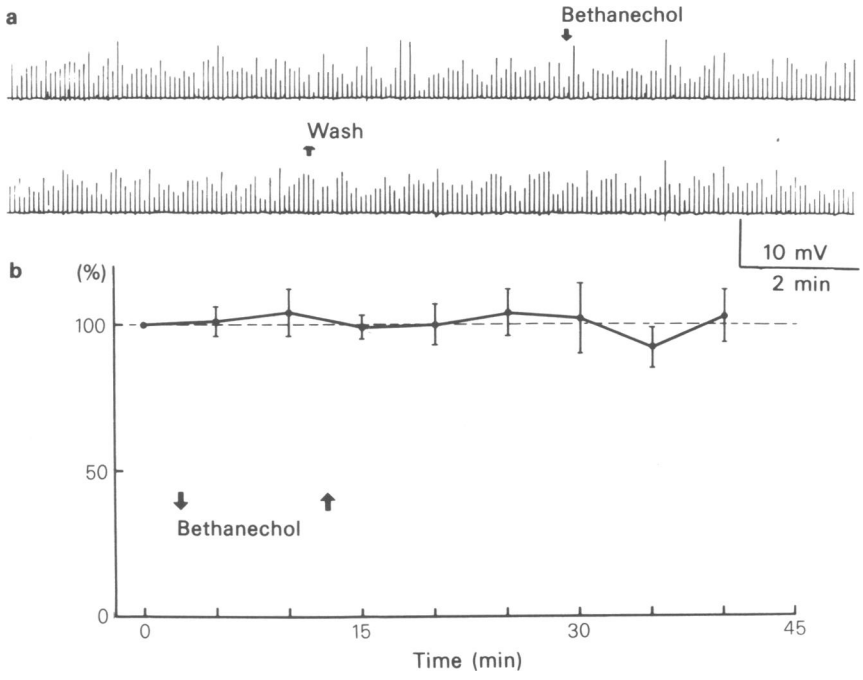

Figure 4 (a) Effect of bethanechol 10 μM, on fast e.p.s.ps in a low Ca^{2+}-high Mg^{2+} solution containing 3 μM atropine. Arrows indicate the presence of bethanechol in the bathing solution. (b) Effect of bethanechol (10 μM) on the mean quantal content of fast e.p.s.ps. Ordinates show the percentage change in the mean quantal content of fast e.p.s.ps; mean values are shown and vertical lines indicate s.e.mean ($n = 7$). Arrows indicate the period of application of bethanechol.

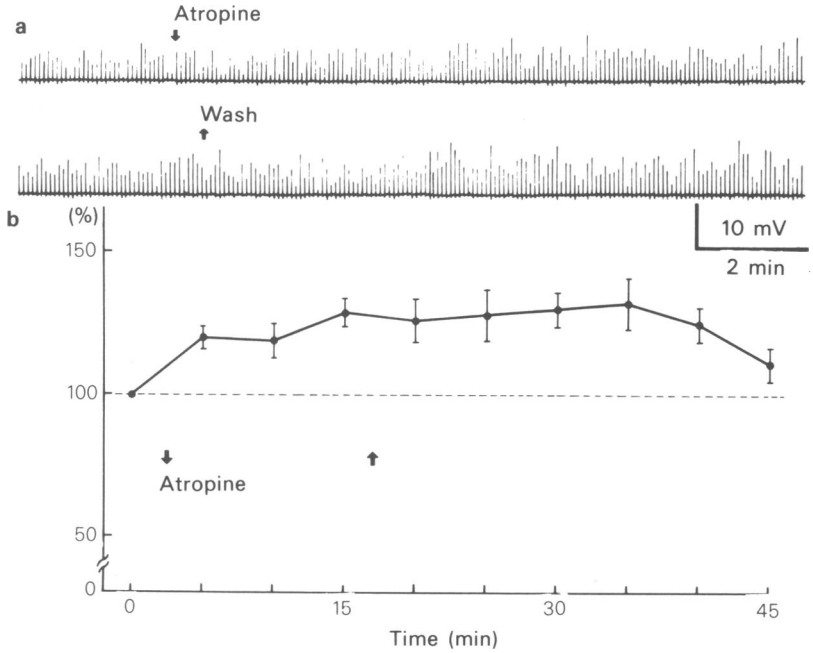

Figure 5 (a) Effect of atropine (3 μM) on fast e.p.s.ps in a low Ca^{2+}-high Mg^{2+} solution. Arrows indicate the period when atropine was present in the bathing solution. (b) Effect of atropine (3 μM) on the quantal content of fast e.p.s.ps. Ordinates show the percentage change in the mean quantal content of fast e.p.s.ps; mean values are shown and vertical lines indicate s.e.mean ($n = 14$). Arrows indicate the period of application of atropine.

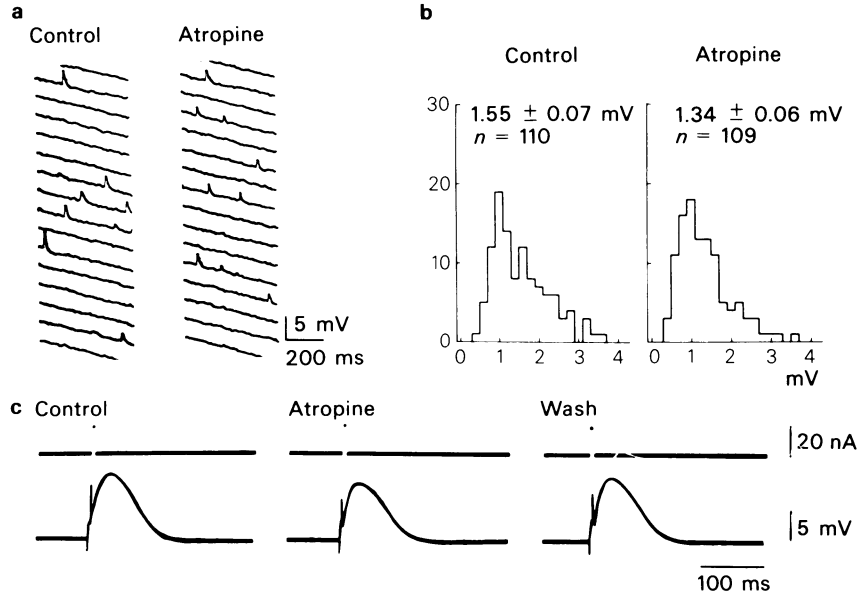

Figure 6 (a) Miniature e.p.s.ps before and during application of atropine (3 μM). (b) Histograms showing amplitude of miniature e.p.s.ps before (10 min) and during (10 min) application of atropine. Ordinates indicate numbers of miniature e.p.s.ps and abscissae the amplitudes. The mean amplitude ± s.e. is shown and n is the number of miniature e.p.s.ps during 10 min. (c) Effect of atropine (3 μM) on potential induced by iontophoretic application of acetylcholine before, during and after application of atropine. Upper and lower traces are the currents for iontophoresis and membrane potential, respectively.

$129.2 \pm 5.0\%$ ($n = 14$) of the control value 15 min after an application of bethanechol (3 μM). The mean amplitude of miniature e.p.s.ps and the peak amplitude of ACh-potentials were decreased to $86.4 \pm 4.2\%$ ($n = 3$) and $91.7 \pm 5.3\%$ ($n = 5$), respectively, in the presence of 3 μM atropine.

Effect of bethanechol on fast e.p.s.ps in the presence of α-adrenoceptor blocking agents

The effect of α-adrenoceptor blocking agents (phenoxybenzamine and phentolamine) on the depressant action of bethanechol on the active release of ACh was examined (see Discussion). A ganglion initially immersed 30 min or more in a low Ca^{2+}-high Mg^{2+} solution containing an α-adrenoceptor blocking agent was then immersed in 10 μM bethanechol. The mean quantal content of fast e.p.s.ps decreased to $74.4 \pm 6.9\%$ ($n = 8$) of control during application of 10 μM bethanechol in the presence of 10 μM phenoxybenzamine and to $47.5 \pm 5.5\%$ ($n = 4$) of the normal during an application of 10 μM bethanechol in the presence of 10 μM phentolamine.

Discussion

The present experiments demonstrate the fast e.p.s.ps are depressed by the action of bethanechol at concentrations which are too small to cause depolarization of the ganglion cell in low Ca^{2+}-high Mg^{2+} solution, or Ringer solution containing (+)-Tc. Such a depressant action of bethanechol on fast e.p.s.ps was completely prevented in the presence of atropine, indicating that the depressant action is a muscarinic action of bethanechol. Two possible explanations can be considered for this depressant action of bethanechol on fast e.p.s.ps: first, bethanechol may depress the active ACh-release from preganglionic nerve terminals; and second, bethanechol may depress the sensitivity of the subsynaptic nicotinic receptor. The first possibility of a depressant action is supported by the present results showing that the quantal content measured by the variance method was significantly reduced in the presence of bethanechol. Regarding the second possibility, it is difficult to believe that bethanechol depresses the sensitivity of the nicotinic receptor. Nevertheless, this might happen in either of the following ways: bethanechol may have a nicotinic action by depressing the sensitivity of nicotinic receptors on which atropine acts as a nonspecific blocker (MacDermott, Connor, Dionne & Parsons, 1980; Yamada, 1981), or it may depress the sensitivity by its muscarinic action. These possibilities were eliminated by the present results showing that the amplitudes of miniature e.p.s.ps as well as the ACh-potential induced by iontophoresis of ACh are not affected by the action of 10 μM bethanechol. On the basis of these results, it appears that the presynaptic muscarinic receptor inhibits the active ACh-release from nerve terminals in the bullfrog sympathetic ganglion. The fact that the active release of ACh from nerve terminals in the present preparation is facilitated by the action of atropine (3 μM) also supports this conclusion.

The fact that the membrane of preganglionic nerve terminals was not depolarized by the action of bethanechol in a high concentration (1 mM), whereas it was markedly depolarized by the actions of ACh or carbachol in the same concentration, indicates that bethanechol indeed has a specific muscarinic action and is not acting on presynaptic nicotinic receptors (Koketsu & Nishi, 1968; Ginsborg, 1971). Presumably, bethanechol acts on the presynaptic muscarinic receptor which does not cause depolarization of sympathetic preganglionic nerve terminals.

Sympathetic ganglia have small, intensely fluorescent, cells (SIF cells) which contain catecholamines and may possess muscarinic receptors (Eccles & Libet, 1961). A possibility therefore remains that reduction of ACh-release may be caused by the action of catecholamine on preganglionic nerve terminals. Indeed, catecholamine would be released from the SIF cells which are depolarized by bethanechol and would thereby reduce the ACh-release from presympathetic ganglionic nerve terminals via α-adrenoceptors (Christ & Nishi, 1971a, b; Kuba, Kato, Kumamoto, Koketsu & Hirai, 1981). However, the present results make this unlikely as it was shown that the reduction of quantal content during application of bethanechol is maintained in the presence of α-adrenoceptor blocking agents (10 μM phenoxybenzamine or 10 μM phentolamine). It is also of note that the frequency of miniature e.p.s.ps is not affected by 10 μM bethanechol, whereas it is decreased by catecholamine (Christ & Nishi, 1971b; Kuba *et al.*, 1981).

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