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A NOTE ON THE EFFECT OF DITHIOTHREITOL (DTT) ON THE DEPOLARIZATION OF ISOLATED SYMPATHETIC GANGLIA BY CARBACHOL AND BROMO-ACETYLCHOLINE

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The S–S reducing agent, dithiothreitol (DTT) altered the properties of nicotinic receptors in rat superior cervical ganglia such that (i) carbachol became less active as a depolarizing agent and (ii) bromo-acetylcholine produced an irreversible depolarization. The latter was temporarily annulled by hexamethonium (which retained antagonist properties), but returned when hexamethonium was removed. It is concluded that ganglionic nicotinic receptors might be quite similar to those for mono-quaternary agonists in leech dorsal muscle.

Introduction Reduction of disulphide bonds to free sulphydryl (–SH) groups with dithiothreitol (Cleland, 1964) has some interesting effects on the responses of electroplax and striated muscle to acetylcholine-receptor ligands. Reported effects include: (i) reduced affinity of acetylcholine and carbachol; (ii) generation of irreversible agonist activity with halogenated acetylcholine compounds; and (iii) generation of an agonist response to hexamethonium (Karlin & Bartels, 1966; Karlin & Winnik, 1968; Albuquerque, Sokoll, Sonneson & Thesleff, 1968; Mittag & Tormoy, 1970; Rang & Ritter, 1971; Ross & Triggle, 1972; Ben-Haim, Landau & Silman, 1973; Ben-Haim, Dreyer & Peper, 1975).

The objective of the present experiments was to find out whether ganglionic nicotinic receptors showed any or all of these changes after S–S reduction with DTT, as an aid to the eventual definition of their relationship with other nicotinic receptors.

Methods Demarcation potentials between ganglion and postganglionic nerve trunk were recorded from isolated superior cervical ganglia of the rat with extracellular electrodes, using a continuous superfusion technique (Brown & Marsh, 1975). The superfusion fluid consistent of Krebs solution at 25°C containing hyoscine 1 μM (to annul effects on muscarinic receptors) and physostigmine 10 μM (to prevent hydrolysis of bromo-acetylcholine; Chiou & Rama Sastry, 1968). The normal pH of the bicarbonate-buffered solution bubbled with 95% O_2 and 5% Co_2 was 7.4; during application of DTT a Tris-buffered solution at pH 8 was used.

Drugs. Dithiothreitol (DTT) and 5'5-dithio-*bis* (2-nitrobenzoic acid) (DTNB) were obtained from Sigma. Bromo-acetylcholine (BAC) was synthesized and purified as the perchlorate salt according to the method of Chiou & Rama Sastry (1968); a second sample was kindly provided by Professor Chiou.

Results

Action of bromoacetylcholine In the normal ganglion (i.e. without prior S–S reduction) BAC produced a readily-reversible depolarization; in contrast to the observation of Chiou (1974) on skeletal muscle, no evidence for irreversibility was obtained after contact times up to 20 minutes. Compared with carbachol, BAC was (i) slightly (1.5–2 times) more effective (in the presence of physostigmine) and (ii) tended to show greater desensitization (Figure 1).

Effect of dithiothreitol DTT was applied for 15 min at pH 8, and was then washed out for 10 min with normal Krebs solution at pH 7.4 before testing the agonist. DTT itself produced small and inconsistent changes in the resting demarcation potentials.

(a) Carbachol—DTT (1 mM) shifted the carbachol dose-response curve to the right by some 0.8 log units; subsequent exposure to 3 mM DTT produced a slight further shift. This depression persisted for several hours after DTT, and was fully reversed by 10 min reoxidation with 1 mM DTNB.

(b) BAC—After DTT, the depolarization produced by BAC became irreversible, i.e. polarity was not restored on washing out the BAC with Krebs solution (Figure 1). Depolarization was reversed temporarily by hexamethonium, but returned on washing out the hexamethonium. Even after overnight washing in Krebs solution after a single 2 min application of BAC, addition of hexamethonium still produced a hyperpolarization, suggesting that the depolarization was very persistent. With continuous recording the level of depolarization tended to diminish gradually, but this might have been due to spread of depolarization to the reference electrode on the postganglionic nerve trunk, consequent upon a discharge of the ionic gradients (cf. Brown,

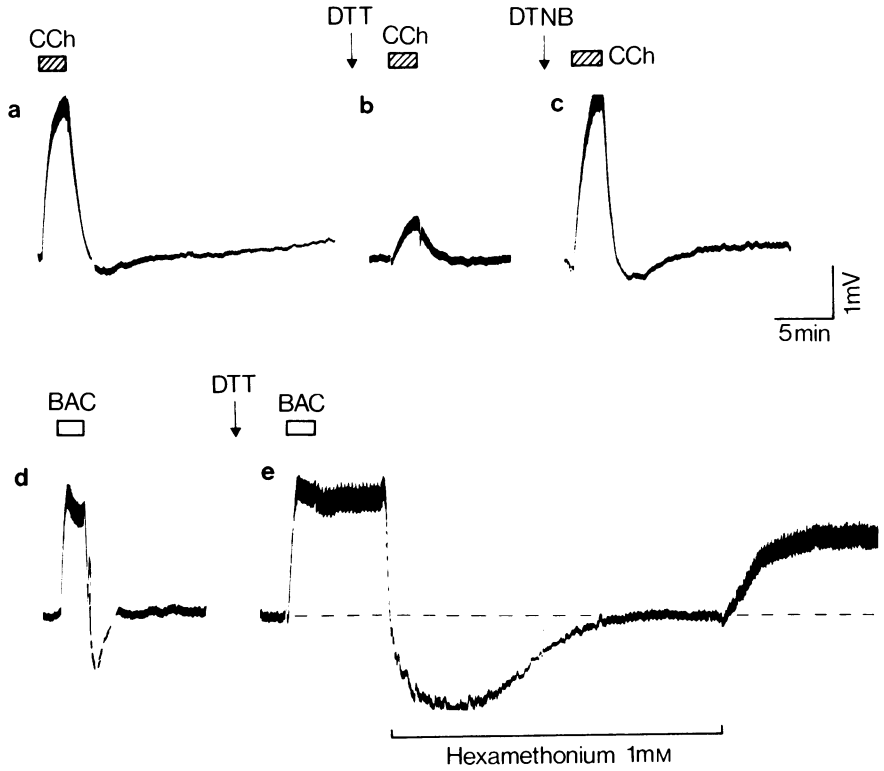

Figure 1 Effects of dithiothreitol (DTT, 1 mM, added for 15 min at the arrows and then washed out) on depolarizing responses to carbachol (CCh, 55 μM) and bromo-acetylcholine (BAC, 32 μM) recorded potentiometrically. The reversible response to carbachol (a) was reduced after DTT (b) and restored after 10 min application of 5'5-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) (c). After DTT (e), the depolarization produced by BAC which was normally reversible (d) persisted when the BAC was washed out, was reversed on addition of hexamethonium 1 mM, but reappeared when the hexamethonium was washed out. The post-agonist hyperpolarizations are due to electrogenic extrusion of accumulated Na^+ ion (Brown *et al.*, 1972). This is very pronounced on addition of hexamethonium during post-DTT BAC-depolarization; the projected baseline demarcation potential in the absence of electrogenic hyperpolarization is indicated by the dashed line. Agonists were added for 2 min at 30 min intervals. Scales: 1 mV, 5 minutes.

Brownstein & Scholfield, 1972; Brown & Scholfield, 1974). In Figure 1, the reversal of potential during exposure to hexamethonium is due to the electrogenic extrusion of accumulated Na^+ ion, see Brown *et al.* (1972); the very prolonged hyperpolarization after BAC would reflect the large Na^+ accumulation during the irreversible depolarization.

Three other features of post-DTT BAC depolarization may be noted. (i) The desensitization was much less pronounced. (ii) The magnitude of the depolarization was still dependent upon the concentration of BAC, such that step-wise increments in the depolarization occurred with cumulative addition of BAC. The sensitivity of the preparation to BAC, as judged from these increments, appeared to be greater after DTT, although the peak magnitude of the depolarization was usually less than that observed before DTT. (iii) Carbachol, superimposed on a BAC-depolarization, produced a reversible increment in depolarization; even when additional BAC itself had no further effect. This suggests that not all of the receptors were permanently affected by 1 mM DTT.

(c) Hexamethonium—Hexamethonium retained its normal antagonistic action (as is apparent in Figure 1) and showed no evidence of agonist action. We have not yet determined whether its affinity constant is changed.

Discussion

These experiments are essentially qualitative; further quantitation of (e.g.) changes in apparent dissociation constants are clearly called for, together with

measurements of conductance changes to obviate secondary effects of the undoubtedly-substantial changes in ionic gradients (cf. Brown & Scholfield, 1974). Nevertheless, certain points of interest emerge.

Firstly, there is a clear similarity between the effects of DTT on the actions of carbachol and BAC on the ganglion and those previously reported for electroplax by Silman & Karlin (1969). The effect on carbachol is compatible with a reduction in affinity constant. With respect to the irreversible effect of BAC, Silman & Karlin (1969) postulated the presence of an S–S group adjacent to the anionic subsite of the receptor with which the quaternary head-group of BAC interacts. After reduction of the S–S bond to free –SH groups, interaction with the anionic subsite facilitates a covalent reaction between the –SH group and the bromo-acetyl moiety, which in turn sustains the interaction of the quaternary head-group with the anionic subsite. Addition of hexamethonium blocks the latter without disrupting the covalent link, so that renewed depolarization occurs on washing out the hexamethonium. It remains unclear why the irreversible reaction should not go to completion in the continued presence of BAC, instead of attaining a concentration-dependent ceiling.

Secondly, a point of difference from both electroplax and chick biventer muscle emerges in that, in the ganglion, hexamethonium showed no clear agonist action after DTT (cf. Karlin & Winnik, 1968; Rang & Ritter, 1971). This suggests a closer resemblance of the ganglionic nicotinic receptor to that for mono-quaternary nicotinic agonists in leech dorsal muscle (Flacke & Yeoh, 1968; Ross & Triggle, 1972). This analogy is strengthened by observations that α-bungarotoxin is ineffective on both carbachol receptors in leech muscle (Ross & Triggle, 1972) and on ganglionic nicotinic receptors (Magazanik, Ivanov & Lukomskaya, 1974; D.A. Brown, unpublished observations), in contrast to its well-known action on electroplax and vertebrate striated muscle.

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