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EXPERIMENTS ON THE MECHANISM OF ACTION OF CHLOROCRESOL AND CAFFEINE

BY

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Phenols are known to cause contraction of skeletal muscle. Thus Torda & Wolff (1945a) reported that phenol (1 mg/ml.) caused a contraction of the frog rectus abdominis muscle, and Cori & Cori (1936) and Barnes, Duff & Threlfall (1955) showed that 2,4-dinitrophenol contracted frog skeletal muscle, and the latter also showed that this substance caused a contraction of the isolated diaphragm which was not abolished when the muscle was depolarized with potassium sulphate. Chlorinated phenols, containing from two to five chlorine atoms, cause a contraction of the isolated diaphragm of the rat (Farquharson, Gage & Northover, 1958).

The present experiments locate the site of contraction of chlorocresol in the skeletal muscle of the frog to an energy transfer system probably involving calcium ions, and compare its action with that of caffeine which causes contraction without affecting the membrane potential of the muscle cell (Axelsson & Thesleff, 1958), by a process probably involving the release of calcium ions (Bianchi, 1961; Frank, 1962).

METHODS

The rectus abdominis muscle from the frog (*Rana temporaria* or *esculenta*) was mounted in a 2-ml. organ-bath containing frog-Ringer solution at room temperature (18 to 22° C) bubbled with oxygen, and arranged to record longitudinal isotonic contractions. The lever had a magnification of five-times and a load of 0.6 to 1.0 g. The frog-Ringer solution had the following composition (g/l.): NaCl 6.54, KCl 0.18, $NaHCO_3$ 0.20, NaH_2PO_4 0.001, $CaCl_2$ 0.12 and glucose 2.00.

Dose/response relations were obtained for the agonists studied and the responses were repeated in the presence of antagonists, after the preparation had been immersed for 30 min in frog-Ringer solution containing them. Maximal contractions were obtained to acetylcholine (500 μg/ml.).

Acetylcholine chloride, caffeine base, chlorocresol, *NN*-di-isopropylphosphodiamidic fluoride (mipafox), dyflos, sodium edetate, fructose 1,6-diphosphate, gallamine triethiodide, glucose 1-phosphate, iodoacetic acid, potassium chloride, potassium cyanide, procaine hydrochloride, sodium fluoride and tubocurarine hydrochloride were used. All drugs are expressed in μg of base per ml. of frog-Ringer solution, except mipafox which is expressed as the salt.

RESULTS

Action of chlorocresol. Chlorocresol caused a slow contraction of the rectus abdominis muscle. The threshold was about 50 μg/ml., and there was a good dose/response relationship, but contractions to concentrations of chlorocresol higher than 500 μg/ml. were followed by incomplete relaxations; washing the preparation repeatedly with fresh frog-Ringer solution for 30 min did not always ensure relaxation (Fig. 1), though further washing pro-

duced complete relaxation to the base-line. Contractions to chlorocresol could be obtained for at least 6 hr with only a small decrease in the size of the responses.

Action of tubocurarine or gallamine. Neither tubocurarine (10 μg/ml.) nor gallamine (50 μg/ml.) significantly reduced the size of the responses to chlorocresol (Fig. 2).

Action of anticholinesterases. The size of the contractions to chlorocresol was not altered by treating the preparation with either dyflos (20 μg/ml.) or mipafox (100 μg/ml.) for a period of 1 hr.

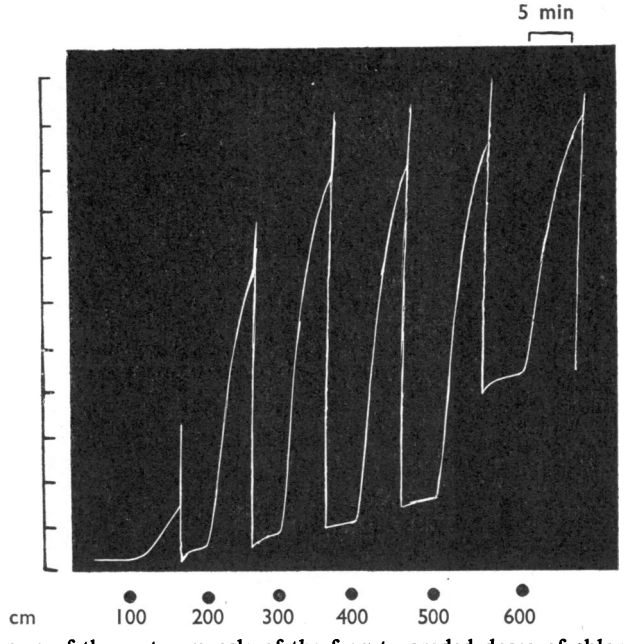

Fig. 1. The responses of the rectus muscle of the frog to graded doses of chlorocresol in frog-Ringer solution. A graded dose/response effect is seen in the range 100 to 300 μg/ml.; after 500 and 600 μg/ml. relaxation was incomplete after washing for 30 min. Further washing allowed relaxation to the base-line.

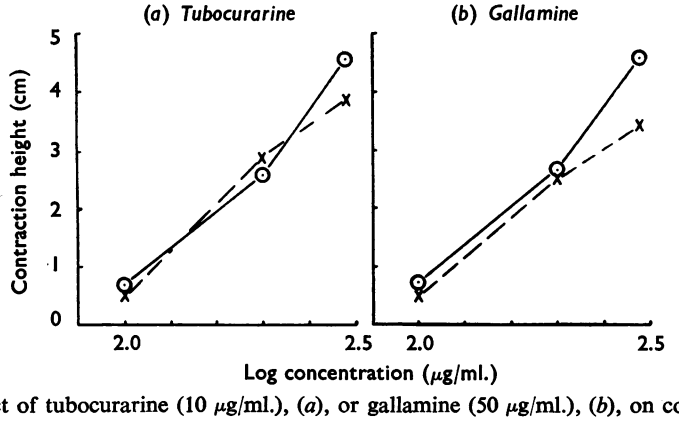

Fig. 2. The effect of tubocurarine (10 μg/ml.), (*a*), or gallamine (50 μg/ml.), (*b*), on contractions of the frog rectus muscle to chlorocresol (abscissa: log concentration). Responses in normal frog-Ringer solution are shown by continuous lines, responses in the presence of the antagonist by the broken lines. The responses were not affected.

" Choline-Ringer " solution. Muscles rendered inexcitable to electrical stimulation or acetylcholine by soaking in frog-Ringer solution in which all the sodium chloride had been replaced by isotonic choline chloride still responded to chlorocresol. The responses were not significantly different from those obtained in normal frog-Ringer solution (Fig. 3).

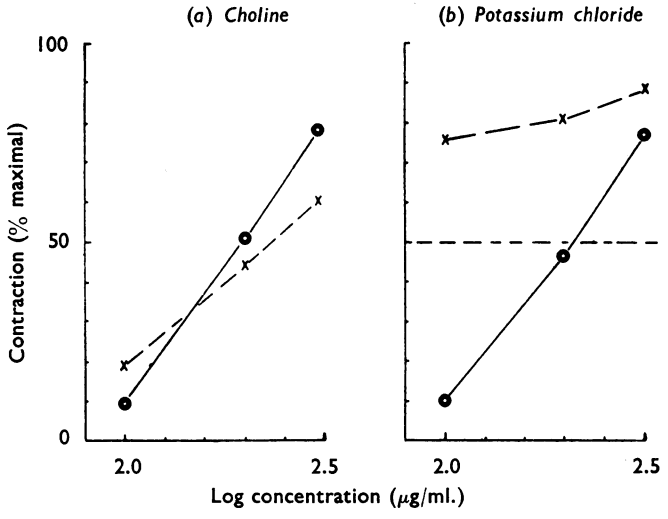

Fig. 3. The effect of " choline-Ringer " (*a*) or isotonic potassium chloride (*b*) on the contractions of the frog rectus muscle to chlorocresol (abscissa: log concentration). The muscles took up a new base-line in isotonic potassium chloride shown by the horizontal broken line. Responses in normal Ringer are shown by the continuous lines, responses in modified Ringer by the broken lines. The responses to chlorocresol were unchanged in " choline-Ringer " and were not abolished in isotonic potassium chloride. Each curve represents the mean of four experiments.

Isotonic potassium chloride. Chlorocresol (50 to 150 μg/ml.) still produced contractions of rectus muscles immersed in isotonic potassium chloride (Fig. 3). Successive contractions to the same concentration of chlorocresol decreased rapidly in size until the muscle no longer responded to chlorocresol; caffeine (1 to 2 mg) was then unable to produce a contraction. Soaking the muscle in normal frog-Ringer solution partially restored the responses to both caffeine and chlorocresol (Fig. 4). Similar results were obtained using isotonic potassium sulphate solution.

Calcium-free frog-Ringer solution containing sodium edetate. Muscles soaked in edetate (750 μg/ml.) for 16 hr would not respond to either chlorocresol or caffeine. Soaking in normal frog-Ringer for 30 min failed to restore the responses to either drug. Muscles soaked in edetate (75 μg/ml.) for 16 hr did not respond to chlorocresol (100 to 300 μg/ml.) or to caffeine (0.5 to 1.5 mg/ml.). Soaking in normal frog-Ringer for 30 min partially restored the responses to chlorocresol and caffeine.

Action of procaine. Procaine (100 μg/ml.) caused only a small reduction in the size of the response to chlorocresol; procaine (500 μg/ml.) caused a slightly larger reduction. Contractions to caffeine were greatly reduced by this concentration of procaine (Fig. 5).

Action of potassium cyanide. Contractions of the rectus muscle to chlorocresol were unaffected by soaking the preparation in potassium cyanide (130 μg/ml.).

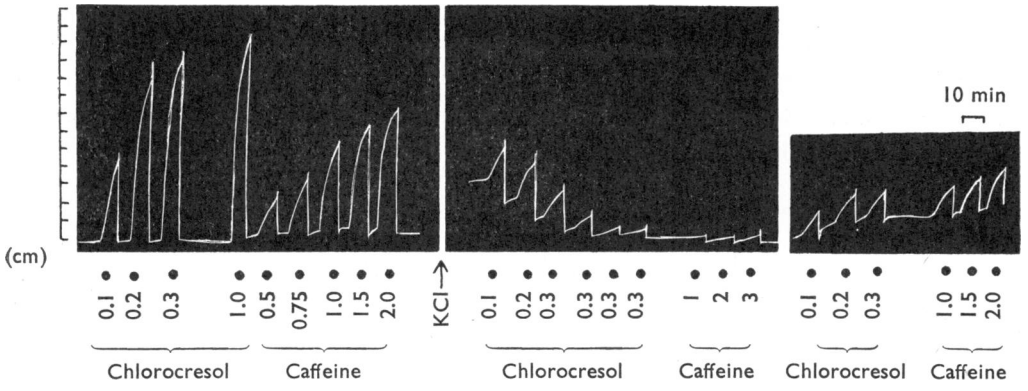

Fig. 4. The effect of isotonic potassium chloride. The two left-hand records show the responses of the rectus to graded doses of chlorocresol or caffeine in normal Ringer, and in isotonic potassium chloride; 30 min between records. The responses to chlorocresol are slowly abolished and the muscle does not contract to caffeine. The right-hand record shows the responses to each drug after the preparation had been washed in normal Ringer solution for 30 min. The responses failed to return to their original size. All doses in mg/ml.

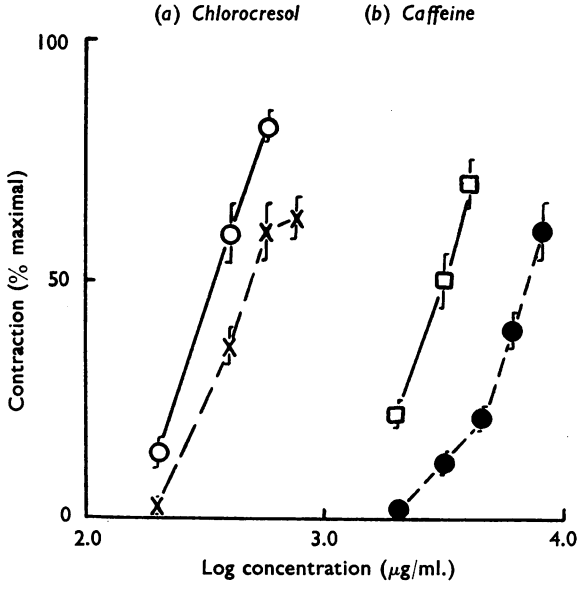

Fig. 5. The effect of procaine (500 μg/ml.) on the responses of the rectus to chlorocresol (*a*) or caffeine (*b*). Responses in normal Ringer solution are shown by the continuous lines, responses in the presence of procaine by the broken lines. Both agonists (abscissa: log concentration) were inhibited by procaine, but caffeine was inhibited more. Each curve, with standard errors, represents the mean of eight experiments.

Action of glucose 1-phosphate. Glucose 1-phosphate (200 and 500 μg/ml.) had little action on the contractions of the rectus muscle to chlorocresol or caffeine.

Action of fructose 1,6-diphosphate. Fructose 1,6-diphosphate (500 μg/ml.) had little action on the contractions of the rectus muscle to chlorocresol or caffeine.

Action of sodium fluoride. Sodium fluoride (42 and 84 μg/ml.) had little effect on contractions of the muscle to chlorocresol or caffeine. Increasing the concentration of the

inhibitor to 210 or 294 μg/ml. reduced the size of the responses to both drugs but the response to caffeine was reduced only at the highest concentration used. Chlorocresol responses were inhibited at all concentrations (Fig. 6).

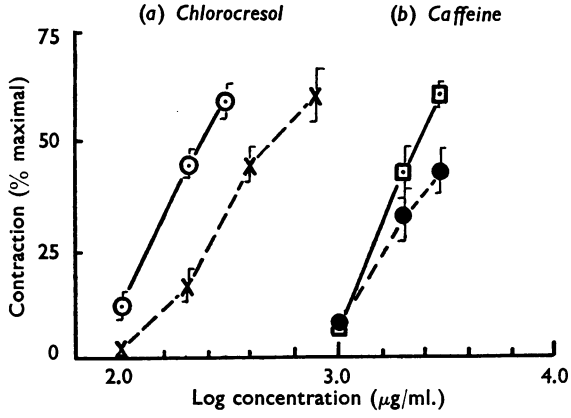

Fig. 6. The effect of sodium fluoride (294 μg/ml.) on the contractions of the rectus to chlorocresol or caffeine. Responses in normal Ringer are shown by the continuous lines, responses in the presence of the inhibitor by the broken lines. The responses to chlorocresol (abscissa : log concentration) were inhibited by this concentration of sodium fluoride but only the response to the highest dose of caffeine was reduced. Each curve represents the mean of four experiments.

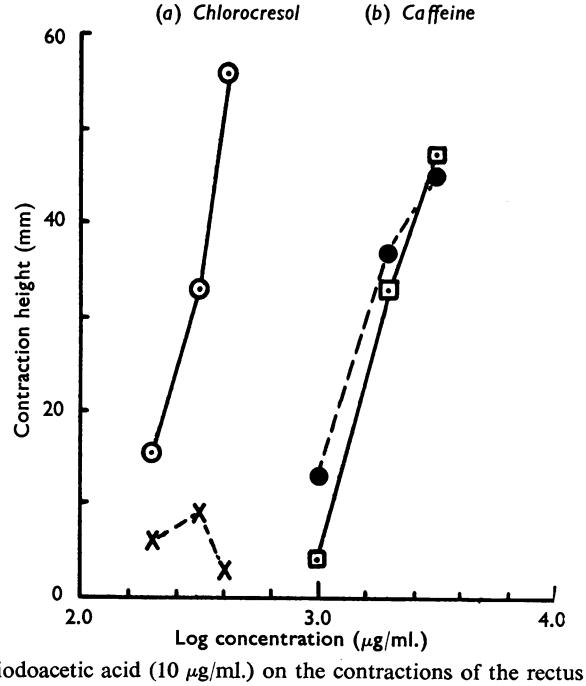

Fig. 7. The effect of iodoacetic acid (10 μg/ml.) on the contractions of the rectus to chlorocresol (*a*) or caffeine (*b*). Responses in normal Ringer solution are shown by continuous lines, responses in the presence of the inhibitor by the broken lines. Abscissa: log agonist concentration. The responses to chlorocresol were blocked by this concentration of iodoacetic acid, but those to caffeine were not affected. Each curve represents the mean of eight experiments.

In experiments conducted under anaerobic conditions in the presence of 2mM-potassium cyanide in frog-Ringer solution gassed with 95% nitrogen and 5% carbon dioxide, both 210 and 294 μg/ml. of sodium fluoride sent the muscle into spasm within 30 min.

Action of iodoacetic acid. Iodoacetic acid (5, 8 and 10 μg/ml.) abolished the contractions of the rectus muscle to chlorocresol. The responses to caffeine were unaffected (Fig. 7). Iodoacetate in the concentrations used had no contractile action on the muscle.

Effect of chlorocresol on responses to caffeine. Chlorocresol (1 and 5 μg/ml.) increased the size of the responses of the rectus muscle to caffeine (Fig. 8). The effect was most marked at low concentrations of caffeine, the threshold concentration to caffeine being approximately halved.

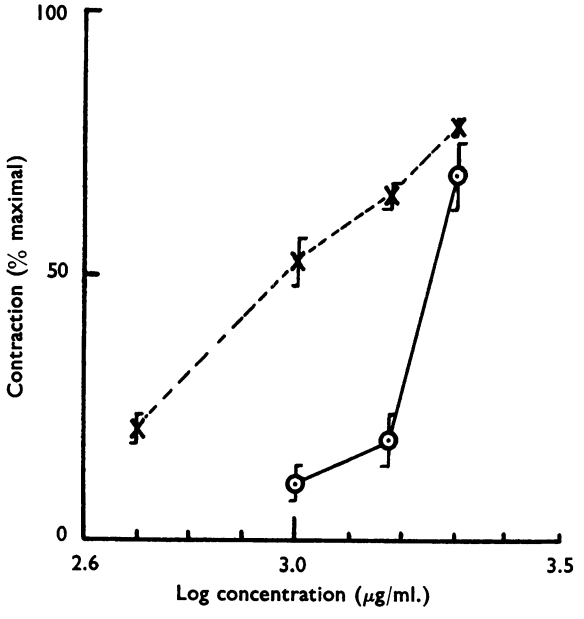

Fig. 8. The effect of chlorocresol (5 μg/ml.) on contractions of the rectus to caffeine (abscissa: log concentration). Responses in normal Ringer solution are shown by the continuous lines; responses in the presence of chlorocresol by the broken lines. Chlorocresol sensitized the muscle to caffeine. Each curve, with standard errors, represents the mean of five experiments.

DISCUSSION

The observation that chlorocresol produced graded contractions of the frog rectus muscle which were not blocked by tubocurarine or gallamine or enhanced by cholinesterase inhibitors suggested that the contractions did not involve an action on an acetylcholine receptor leading to a depolarization of the muscle cell membrane. Supporting evidence for this was provided by the ability of chlorocresol to cause contractions in muscles rendered unresponsive to electrical stimulation or acetylcholine after immersion in " choline-Ringer," or in muscles depolarized by potassium ions. It appeared that the action of chlorocresol in causing a contraction was independent of a mechanism involving depolarization of the muscle cell membrane.

Current concepts of the mechanism of muscle contraction suggest that calcium ions play an essential part in initiating contraction (Shanes, 1958; Bianchi & Shanes, 1959; Weber & Winicur, 1961) and, although chlorocresol still caused contraction in isotonic potassium chloride or in calcium-free frog-Ringer solution containing sodium edetate, the muscle was shown to be ultimately dependent on external calcium ions for the continued production of contractions. Similar results were obtained with caffeine. Kutscha (1961) also demonstrated that contractures of frog sartorius muscle to caffeine or dinitrophenol were dependent upon the extracellular calcium concentration and Frank (1962, 1963), using a different method, reported similar results for caffeine on the frog toe muscle but with more complete recovery of the response than seen in the present experiments.

Procaine and other local anaesthetics in high concentrations inhibit caffeine-induced contractions of frog muscle (Schuller, 1925; Straub & Domenjoz, 1941; Hardt & Fleckenstein, 1949) by a competitive action (Feinstein, 1963). Procaine (500 μg/ml.) was found to inhibit caffeine-induced contractions of the rectus muscle more than it inhibited chlorocresol, which suggested that caffeine and chlorocresol differed in their mechanism of action. Feinstein (1963) demonstrated that the enhanced efflux and influx of calcium caused by caffeine in frog muscle (Bianchi, 1961) was much reduced by local anaesthetics.

Nitro- and chlorophenols can uncouple oxidative phosphorylation both *in vitro* and *in vivo* (Simon, 1953; Brodie, 1955; Parker, 1958; Slater, 1962). Thus it became important to enquire whether chlorocresol might have a metabolic action linked with its ability to cause contraction. This was investigated by using enzyme inhibitors. Potassium cyanide, in a concentration (130 μg/ml.) known to inhibit oxidative processes dependent on molecular oxygen in frog muscle (Carey, Conway & Kernan, 1959), had little action on contractions of the rectus muscle to chlorocresol. However, iodoacetic acid, an inhibitor of oxidative processes in the pyruvic acid cycle, inhibited contractions to chlorocresol but, in contrast, those to caffeine were unaffected. Iodoacetate inhibits the breakdown of glucose at the stage of conversion of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid, and this suggested that the effect of chlorocresol arose from an interference with metabolism at this or a later stage in the glycolytic cycle. It was observed that glucose 1-phosphate and fructose 1,6-diphosphate did not modify the contractions to chlorocresol, in concentrations which increased the amplitude of beat of the frog heart (Freeman, 1930; Lindner & Rigler, 1931) or of the rabbit heart (Gialdroni-Grassi, 1957); they also increased the response of the frog rectus muscle to acetylcholine (Torda & Wolff, 1945b; Stepanenko & Silaeva, 1959) and enhanced the contractions of the rat diaphragm (Ellis, 1956). This adds confirmatory evidence to the suggestion that the action of chlorocresol probably occurs after these two stages in the glycolytic cycle.

Sodium fluoride, another inhibitor, believed to act on the second energy producing reaction of the pyruvic acid cycle, also inhibited selectively contractions due to chlorocresol. Therefore, chlorocresol can probably inhibit oxidative phosphorylation in the rectus muscle since its action may be blocked by iodoacetate in low concentrations and inhibited by fluoride. The use of iodoacetate and fluoride as enzyme inhibitors separated the site of action of chlorocresol from that of caffeine. It should be noted in passing that the action of chlorocresol may not necessarily be confined to the energy transfer reactions of the glycolytic cycle since these all involve an intermediate containing a sulphydryl group (Slater, 1962), of which iodoacetate is a known inhibitor. I conclude that chlorocresol

contracted the rectus muscle by an action involving an energy transfer reaction, and also probably the binding or release of calcium ions.

Turning now to the action of caffeine, it is noteworthy that this was not modified by any antagonist except procaine. Of interest is the sensitization of the muscle to caffeine by low concentrations of chlorocresol which had no contractile activity. These experiments support the views of others (Bianchi, 1961; Feinstein, 1963) that caffeine acts directly on the muscle and that calcium ions are involved.

SUMMARY

1. Dose/response measurements were made on the frog rectus abdominis muscle with chlorocresol or caffeine as agonists.

2. Contractions due to chlorocresol were neither blocked by tubocurarine or gallamine, nor enhanced by the anticholinesterase drugs, *NN*-di-isopropylphosphodiamidic fluoride (mipafox) or dyflos, nor abolished when the muscle was depolarized by potassium ions or made inexcitable by lack of sodium ions.

3. Procaine antagonized caffeine more effectively than chlorocresol, and responses to both agonists were abolished by prolonged soaking in calcium-free frog-Ringer solution, suggesting that calcium ions are involved in the responses to both agonists.

4. Iodoacetic acid or sodium fluoride selectively antagonized chlorocresol, but not caffeine.

5. Glucose 1-phosphate or fructose 1,6-diphosphate had no effect on the responses to either agonist, and responses to chlorocresol were not affected by potassium cyanide.

6. Chlorocresol in low concentrations sensitized the muscle to caffeine.

7. It is concluded that chlorocresol caused contraction by inhibiting an energy transfer process in the muscle, probably involving the binding or release of calcium in the muscle cell.

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