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A FURTHER INVESTIGATION INTO THE ENERGY DEPENDENCE OF ANGIOTENSIN II-INDUCED CONTRACTIONS OF ISOLATED SMOOTH MUSCLE PREPARATIONS

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1 The energy dependence of angiotensin and acetylcholine-induced contractions of rat descending colon and uterus was investigated.

2 Differences were observed in the effect of anaerobic substrate depletion upon responses of colon and oestrous and dioestrous uterus. These were attributed to differences in the energy metabolism of the tissues and were correlated with differences in tissue levels of glycogen.

3 The preferential reduction of angiotensin responses of dioestrous uterus and descending colon when exposed to 2,4-dinitrophenol, was evidence for an energy dependent stage in the angiotensin response distinct from the contraction process itself.

4 The absence of a preferential reduction of the angiotensin response of oestrous uterus when exposed to 2,4-dinitrophenol appeared to be related to the ability of this tissue to generate ATP by anaerobic glycolysis.

5 It was concluded that the energy for the angiotensin response may be derived either anaerobically or aerobically, depending upon the tissue.

Introduction

Angiotensin has been shown to produce a contraction of many smooth muscle preparations (Khairallah, 1971; Gross, 1971). In a previous report, we showed that the response of guinea-pig ileum to angiotensin appeared to involve an energy dependent step distinct from the contraction process (Crocker & Wilson, 1972; 1974). However, the response of this tissue to angiotensin is composed of a direct component and an indirect component through cholinergic nerves that can be blocked by atropine (Khairallah & Page, 1961; Robertson & Rubin, 1962; Godfraind, Kaba & Polster, 1966a, b). In the present study, we have extended these investigations to two smooth muscle preparations where the action of angiotensin has been shown to be wholly direct; rat descending colon (Regoli & Vane, 1964) and rat uterus (Khairallah & Page, 1961).

A preliminary account of some of these findings was communicated in September 1973 (Wilson, Crocker & Willavoys, 1974).

Methods

Preparations

White, Wistar rats (150-200 g) that had been starved for 12 h prior to experimentation were killed by cervical dislocation. Intestinal preparations were taken from the terminal 5 cm of colon immediately adjacent to the rectum and were flushed through with Tyrode solution, prior to mounting in 2.5 cm segments. Uterine preparations consisted of whole uterine horns removed from rats at oestrus or dioestrus, as determined by examination of the vaginal smear.

All preparations were suspended in 20 ml organ baths containing Tyrode solution bubbled with air. Colon segments were maintained at 32°C and uterine preparations at 28°C. The composition of the Tyrode solution was as follows: NaCl 137 (mM), KCl 2.7, $MgSO_4 . 7H_2O$ 1.1, $NaH_2PO_4 . 2H_2O$ 0.42, $NaHCO_3$ 11.9, $CaCl_2 . 2H_2O$ 1.8, glucose 5.6.

In experiments involving anoxia the Tyrode solution was placed under vacuum for 4 h and then the vacuum was replaced by oxygen-free nitrogen. The Tyrode reservoir and the organ bath were then gassed with nitrogen instead of air.

Recordings

It has been reported that isometric recording reveals more information about the individual components contributing to a muscle response

than isotonic recording (Paton, 1961; Paton & Rothschild, 1965). When possible, therefore, we employed Devices 2ST02 isometric transducers with an applied resting tension of 1 g. However, uterine preparations deteriorated rapidly under isometric conditions and therefore these responses were recorded with Devices 2LDO1 isotonic transducers with an applied load of 1 g. Both isotonic and isometric transducers were coupled to Devices M2 recorders.

Tissues were equilibrated for 1.5 h and then the contractile responses were recorded at the maximal sustained deviation from the baseline. A tissue contact time of 90 s was employed for both angiotensin and acetylcholine. In all experiments, concentrations of acetylcholine and angiotensin were selected which produced responses approximately equal to the 50% maximal acetylcholine response.

Glycogen assay

The determination of the glycogen content of colon and uterine muscle samples was carried out with the method of Lo, Russel & Taylor (1970). The assay involved precipitation of tissue glycogen followed by spectrophotometric estimation using a modified phenolsulphuric acid technique and has been reported to be superior to the older anthrone method (Lo *et al.*, 1970). All reagents were of analytical grade and spectrophotometric measurements were made on a Pye Unicam SP500 MkII spectrophotometer.

Drugs and chemicals

The following chemicals were used: angiotensin II (Val 5-angiotensin II, Asp-β-amide; 'Hypertensin', Ciba), acetylcholine chloride (B.D.H.), 2,4-dinitrophenol (Sigma), oyster glycogen (B.D.H.), concentrated sulphuric acid (Analar), potassium hydroxide (Analar), sodium sulphate (Analar), crystalline phenol (Analar), ethyl alcohol, Absolute.

Calculation of results

All results have been expressed as a percentage reduction of the control response taken in normal Tyrode solution gassed with air. The control responses were measured when three successive exposures to the selected concentration of spasmogen had induced equal sized contractions. For each treatment, the reported results are the means of values obtained from at least six tissues. The effects of the experimental treatments have been analysed using Student's *t* test.

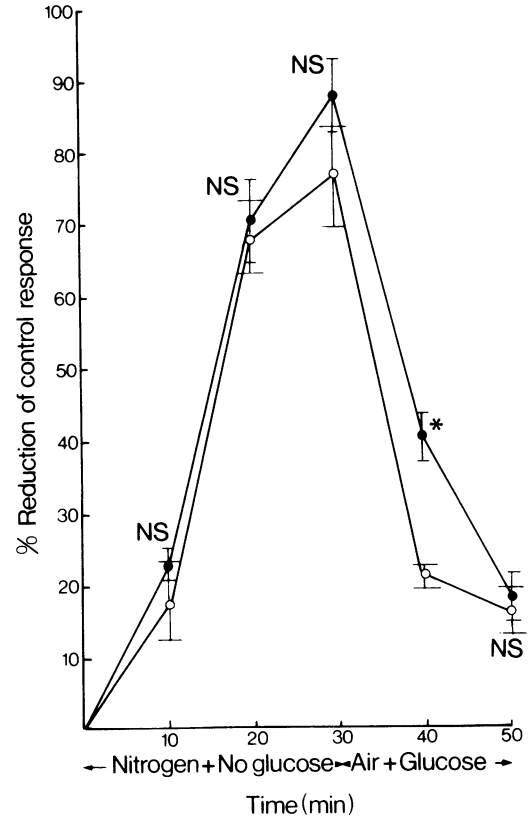

Figure 1 The effect of combined glucose removal and anoxia upon the contractile responses of rat descending colon to angiotensin and acetylcholine. (○) and (●) denote the mean percentage reduction (with s.e. mean) of the acetylcholine and angiotensin responses respectively. N.S. $P > 0.05$, $^*P < 0.01$.

Results

The effect of glucose lack and anoxia, separately and in combination

Exposure of preparations of rat descending colon and uterus to glucose-free Tyrode solution for periods of up to 2 h failed to affect their responses to either acetylcholine or angiotensin. Their responses to both agonists were also unaffected by exposure to nitrogen for periods of up to 3 hours. Since there was no effect with either treatment, the effect of combining the treatments was investigated.

Figure 1 shows the percentage reductions of angiotensin and acetylcholine responses which occurred during a 30 min exposure of rat

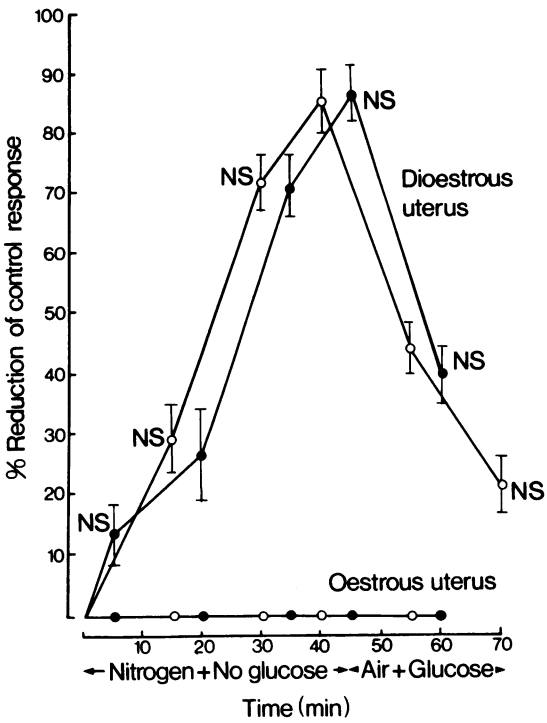

Figure 2 The effect of combined glucose removal and anoxia, upon the contractile responses of oestrous and dioestrous rat uterus to angiotensin and acetylcholine. (○) and (●) denote the mean percentage reduction (with s.e. mean) of the acetylcholine and angiotensin responses respectively. The percentage reduction of the angiotensin response is compared with that of the immediately preceding acetylcholine response. NS $P > 0.05$.

descending colon to glucose-free Tyrode and anoxia. There was a progressive reduction of responses to both agonists but at no time was there a significant difference between the percentage reduction of the angiotensin and acetylcholine responses. After 30 min the percentage reduction of the angiotensin response was 87.5 ± 4.9 ($n = 9$) and that of the acetylcholine response was 76.9 ± 8.1 ($n = 9$) which suggested a marked impairment of the contractile process.

Figure 2 shows the percentage reductions of the angiotensin and acetylcholine responses which occurred during a 45 min exposure of rat dioestrous uterus to glucose-free Tyrode solution and anoxia. With dioestrous uterus the results were similar to those obtained with descending colon (Figure 1). There was a progressive reduction of responses to both agonists with no significant difference between the percentages reduction of the angiotensin responses and the acetylcholine responses. After 40 min, acetylcholine responses were reduced by $85.8 \pm 5.7\%$ ($n = 6$) and after 45 min angiotensin responses were reduced by $86.9 \pm 4.6\%$ ($n = 6$). However, using the same conditions, the response of oestrous uterus to either angiotensin or acetylcholine was unaffected. Prolongation of exposure times for up to 5 h failed to affect the ability of this tissue to respond to either of the two agonists.

Measurement of tissue glycogen content

Although there was no difference in the responses of colon and uterus to acetylcholine compared with angiotensin during metabolic inhibition there was a difference between the responses of oestrous and dioestrous uterus which was significant for both angiotensin ($P < 0.001$) and acetylcholine ($P < 0.001$). Since under these conditions of metabolic inhibition, the tissue can derive energy from anaerobic glycolysis the difference in the responses of oestrous and dioestrous uterus might be due to differences in the levels of tissue glycogen.

The glycogen content of rat oestrous uterus, measured by the method of Lo *et al.* (1970), was 252.0 ± 5.0 mg/100 g wet weight of tissue ($n = 46$) which was significantly higher ($P < 0.001$) than the content of dioestrous uterus 147.0 ± 6.0 mg/100 g wet weight of tissue ($n = 30$). There was no significant difference between the glycogen content of descending colon taken from male rats, 115.0 ± 3.0 mg/100 g wet weight tissue ($n = 15$), from oestrous rats, 112.0 ± 3.0 mg/100 g wet weight of tissue ($n = 46$), or from dioestrous rats, 113.0 ± 2.0 mg/100 g wet weight of tissue ($n = 48$).

The effect of 2,4-dinitrophenol

To investigate further the importance of anaerobic glycolysis as an energy yielding process, experiments were performed using a Tyrode solution containing glucose (5.6 mM) as substrate and 2,4-dinitrophenol (0.1 mM). This concentration of 2,4-dinitrophenol has been shown to be effective in uncoupling oxidative phosphorylation in rat uterine tissue (Rangachari, Paton & Daniel, 1972).

Segments of descending colon from oestrous and dioestrous rats were exposed to this solution for 25 min and responses to acetylcholine and angiotensin were recorded. There was no difference in the responses obtained from dioestrous preparations compared with oestrous preparations but in both the percentage reduction

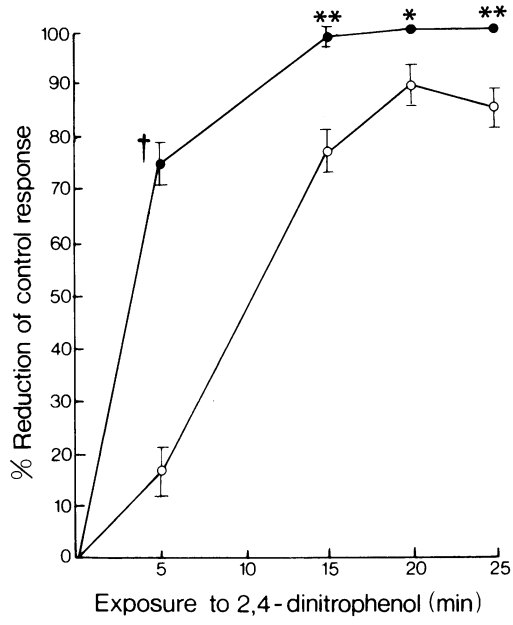

Figure 3 The effect of 2,4-dinitrophenol (0.1 mM) upon the contractile responses of dioestrous rat descending colon to angiotensin and acetylcholine. (○) and (●) denote the mean percentage reduction (with s.e. mean) of the acetylcholine and angiotensin responses respectively. * $P < 0.05$, ** $P < 0.01$, † $P < 0.001$.

of the angiotensin response was significantly greater ($P < 0.001$) than that of the acetylcholine response. Figure 3 shows the rapid increase in the percentage reductions of the responses to both agonists when dioestrous colon was exposed to 2,4-dinitrophenol (0.1 mM) for up to 25 minutes. After 5 min exposure to 2,4-dinitrophenol, the angiotensin response was reduced by 75.2 ± 4.1% ($n = 5$) and the acetylcholine response was reduced by 16.6 ± 4.8% ($n = 5$). The difference between the percentage reduction of the angiotensin response and that of the acetylcholine response was highly significant ($P < 0.001$). Although at 15 min both responses were markedly reduced and the angiotensin response almost abolished, there was still a significant difference ($P < 0.001$) between the percentage reduction of the angiotensin response (98.3 ± 1.6) and that of the acetylcholine response (77.4 ± 4.1). When a lower concentration of 2,4-dinitrophenol was used (0.05 mM) maximal inhibition occurred within 30 min of exposure and the angiotensin response was inhibited by 69.2 ± 2.2% ($n = 9$) and the acetylcholine response by 34.4 ± 4.3% ($n = 9$). The difference between the percentage reduction of the acetylcholine response compared with the angiotensin response was highly significant ($P < 0.001$).

Figure 4 shows the results obtained when

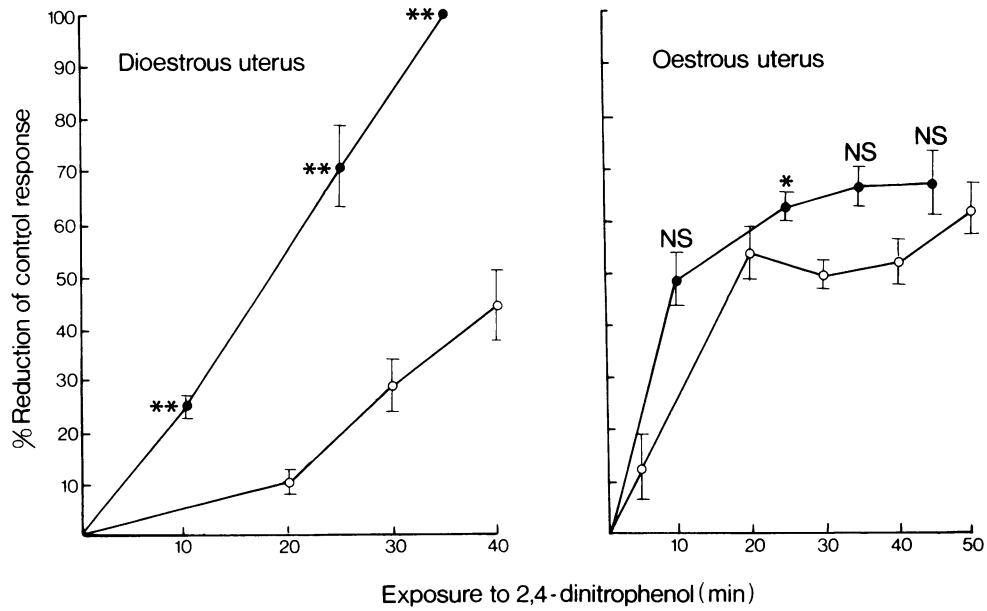

Figure 4 The effect of 2,4-dinitrophenol (0.1 mM) upon the contractile responses of rat oestrous and dioestrous uterus to angiotensin and acetylcholine. (○) and (●) denote the mean percentage reduction (with the standard error of the mean) of the acetylcholine and angiotensin responses respectively. The percentage reduction of the acetylcholine response is compared with that of the immediately preceding angiotensin response. * $P < 0.05$, ** $P < 0.001$.

oestrous and dioestrous uterus were exposed to 0.1 mM 2,4-dinitrophenol. The responses of dioestrous uterus to both angiotensin and acetylcholine were progressively and rapidly reduced during exposure to 2,4-dinitrophenol but at all times the reduction of the angiotensin response was significantly greater ($P < 0.001$) than that of the acetylcholine response. The rate of the reduction of the responses was slower in dioestrous uterus than in descending colon and complete abolition of the angiotensin response on dioestrous uterus was obtained after 35 min compared with 15 min for colon.

The responses of oestrous uterus to both agonists (Fig. 4) showed an initial rapid reduction followed by a slower reduction so that after 30 min exposure to 2,4-dinitrophenol (0.1 mM) the acetylcholine response was reduced by $50.0 \pm 3.3\%$ ($n = 5$) and the subsequent angiotensin response (at 35 min) was reduced by $65.7 \pm 3.4\%$ ($n = 5$). There was no consistent significant difference between the percentage reductions of the angiotensin and acetylcholine responses of oestrus during the period of exposure to 2,4-dinitrophenol (0.1 mM).

Discussion

In a previous study, we investigated the effect of various forms of metabolic inhibition upon responses of guinea-pig ileum to angiotensin and acetylcholine (Crocker & Wilson, 1972, 1974). It was shown that both substrate depletion and anoxia preferentially reduced responses induced by angiotensin and, on the basis of these findings, we proposed the existence of an energy dependent step in the angiotensin response distinct from the contractile process itself and associated with the direct component of angiotensin's action in this tissue. We proposed also that the energy for this step was provided from aerobic metabolism. However, the interpretation of these findings was complicated by the need to use a cholinoceptor antagonist to study the direct effect of angiotensin in guinea-pig ileum so in the present study we have investigated the energy dependence of the angiotensin response in tissues where angiotensin has no indirect, cholinergically, mediated action.

Anoxia and glucose lack separately failed to produce the differential reduction of the angiotensin response of rat colon that had been observed with guinea-pig ileum. Similarly, responses of rat dioestrous and oestrous uterus to angiotensin and acetylcholine were unaffected by anoxia or glucose lack separately. This failure to confirm our previous findings suggested that either our original hypothesis was incorrect or that differences in tissue metabolism made it difficult to demonstrate the same effects. This latter point is supported by many reports in the literature showing that tissues do vary in their susceptibility to metabolic inhibition. Other workers have also observed contractions of isolated smooth muscle preparations both under anaerobic conditions in the presence of glucose (Gross & Clark, 1923; Prasad, 1935; West, Hadden & Farah, 1951; Day & Vane, 1963; Furchgott, 1966; Shibita & Briggs, 1967; Hughes & Coret, 1968; Detar & Bohr, 1972) and under aerobic conditions in the absence of glucose (Prasad, 1935; Furchgott & Wales, 1951; Axelsson, Hogberg & Timms, 1965; Furchgott, 1966; Shibita & Briggs, 1967; Hughes & Coret, 1968; Coe, Detar & Bohr, 1968; Rangachari *et al.*, 1972). Presumably in our experiments enough energy was generated by anaerobic glycolysis of exogenous glucose to support the contractile responses to both agonists under conditions of anoxia or by aerobic metabolism of endogenous glycogen and β-oxidation of fatty acids after removal of glucose from the Tyrode solution.

When anoxia and glucose lack were combined differences were obtained between the responses of the three tissues to both agonists but again no differential reduction of the angiotensin response was observed. The responses of colon and dioestrous uterus to acetylcholine and angiotensin were reduced by the combined treatment whereas the responses of oestrous uterus were unaffected. Again these findings supported the idea that the problem of demonstrating a differential reduction of the angiotensin response might be related to differences in the metabolism of the tissues we were using. It has been shown that isolated smooth muscle preparations derive energy from carbohydrate metabolism and β-oxidation of fatty acids (Furchgott & Shorr, 1946; Coe *et al.*, 1968). It is also well known that concentrations of ATP and creatine phosphate are low in both uterine (Cspao & Gergely, 1950; Walaas & Walaas, 1950; Menkes & Csapo, 1952) and intestinal smooth muscle (Dworaczek & Barrenscheen, 1937; Born, 1956). Therefore, the ability of a preparation to respond under conditions of metabolic inhibition is a reflection of its ability to generate energy by the metabolic pathways operative under those conditions. It has been demonstrated by other workers that many isolated smooth muscle preparations are unable to maintain contractility under conditions of anaerobic substrate depletion (Gross & Clark, 1923; Garry, 1928; Prasad, 1935; West *et al.*, 1951; Furchgott, 1966; Shibita & Briggs, 1967; Rangachari *et al.*, 1972) when the tissue would be dependent upon anaerobic glycolysis of endogenous glycogen.

Therefore, in the present report, the marked

effect of anaerobic substrate depletion on the responses of colon and dioestrous uterus compared with the lack of effect on the responses of oestrous uterus might be a reflection of differences in the energy produced by these tissues from anaerobic glycolysis of endogenous glycogen stores. Thus either oestrous uterus contained a larger amount of glycogen than dioestrous uterus or colon, or the glycolytic pathway was far more active in the oestrous uterus. However, both descending colon and dioestrous uterus were capable of generating sufficient energy to maintain contractions under anaerobic conditions in the presence of exogenous glucose. It seemed probable, therefore, that the activity of the anaerobic glycolytic pathway was not the limiting factor and that the differences in energy production under conditions of anoxia and glucose lack could be attributed to differences in the available glycogen stores.

An analysis of the glycogen content of rat uterus revealed that the glycogen level of oestrous uterus was significantly higher than that of dioestrous uterus. This confirmed several reports that uterine glycogen varied during the oestrous cycle (Boettiger, 1946; West, Jones & Loomis, 1953; West & Cervoni, 1955). The glycogen content of rat descending colon was found to be significantly lower than that of dioestrous uterus but was independent of either the stage of the oestrous cycle or the sex of the animal. Thus oestrous uterus which was unaffected by anaerobic substrate depletion contained significantly more glycogen than either dioestrous uterus or descending colon, both of which were inhibited by anaerobic substrate depletion. Further, descending colon contained significantly less glycogen than dioestrous uterus and was more rapidly inhibited by anaerobic substrate depletion.

Thus we had been able to demonstrate that the differences in the responses of these tissues after metabolic inhibition might indeed be related to differences in their metabolism which in turn might be the reason why we had not confirmed our previous findings (Crocker & Wilson, 1972; 1974). This is illustrated by our present observations that rat descending colon was insensitive to conditions of metabolic inhibition which had reduced the responses of guinea-pig ileum and revealed the differential effect on the angiotensin response. Our findings with colon are supported by reports from several workers that there is a metabolic gradient down the length of the intestine (Alvarez, 1922; Evans, 1923; Prasad, 1935; Dickens & Weil-Malherbe, 1941; Farah, West & Angel, 1950; Sherratt, 1968) and that colon preparations are far less dependent upon oxidative metabolism than preparations of the small intestine (Prasad, 1935; Farah *et al.*, 1935).

In the experiments where preparations were exposed to anoxia, nitrogen was substituted for the normal air supply to the tissues so that the degree of anoxia produced was liable to variation and was probably never complete because of the difficulty of removing all dissolved oxygen from the Tyrode solution. There have been several reports which demonstrated that the effect of anoxia was markedly dependent upon the actual reduction in P_{O_2} achieved (Detar & Bohr, 1968a, b, 1972; Namm & Zucker, 1973). To overcome this problem, a chemical inhibitor of oxidative metabolism, 2,4-dinitrophenol, was used.

2,4-Dinitrophenol has been extensively used to investigate the metabolism of smooth muscle preparations and its effects have been shown to be consistent with its known mode of action as an uncoupler of oxidative phosphorylation (Farah *et al.*, 1950; West *et al.*, 1951; Born & Bulbring, 1955; Daniel, 1964; Rangachari *et al.*, 1972; Greenberg, Wilson & Long, 1973). In our experiments we used 2,4-dinitrophenol in a Tyrode solution containing glucose as an exogenous substrate for glycolysis so that energy production would be dependent upon anaerobic glycolysis and the tissues should be capable of some degree of response to applied spasmogens (Daniel, 1964; Rangachari *et al.*, 1972; Greenberg *et al.*, 1973).

Dinitrophenol progressively reduced the responses of rat descending colon and dioestrous uterus to acetylcholine and angiotensin, but at all times the percentage reduction of the angiotensin response was significantly greater than that of the corresponding acetylcholine response. With oestrous uterus, however, there was a slow reduction of responses to both acetylcholine and angiotensin with no preferential reduction of the angiotensin response. This difference between the effect of dinitrophenol on oestrous uterus and that on dioestrous uterus and descending colon was consistent with the biochemical changes which occur in the uterus during the course of the oestrous cycle. Several workers have reported that anaerobic glycolysis is more efficient in oestrous uterus than in dioestrous uterus (Kerly, 1937; Kerly, 1940; Walaas, Walaas & Loken, 1952). In addition, it is known that the relative efficiencies of anaerobic and aerobic carbohydrate metabolism in uterine tissue varies during the oestrous cycle (Kerly, 1937). Anaerobic metabolism, the most important energy yielding process (West *et al.*, 1953), is at maximum efficiency in the early oestrous uterus when aerobic metabolism is at minimum efficiency. Inhibition of aerobic metabolism would therefore be expected to have less effect upon the overall capacity of oestrous

uterus to generate energy than it would on dioestrous uterus. The capacity of the oestrous uterus to generate ATP anaerobically might then mask the differential reduction of the angiotensin response seen in dioestrous uterus and descending colon.

The reduction of the induced responses of the oestrous uterus by 2,4-dinitrophenol contrasted with the lack of effect produced by combined substrate depletion and anoxia. 2,4-Dinitrophenol was used in the presence of glucose and thus it would be expected that the reduction of responses would be less than the reduction produced by anaerobic substrate depletion, if the dinitrophenol solely acted as an uncoupling agent. There was the possibility that 2,4-dinitrophenol might also interfere with the combination of the agonists with their receptors but this seemed unlikely since we have shown that potassium-induced contractions of these tissues were affected by 2,4-dinitrophenol in the same way (Crocker & Wilson, unpublished observations). Also, other workers have reported an inhibitory action of 2,4-dinitrophenol upon anaerobic contractions of smooth muscle preparations, and this has been attributed to interference with the formation of energy rich phosphate bonds (Farah *et al.*, 1950; West *et al.*, 1951).

The preferential reduction of responses of dioestrous uterus and descending colon to angiotensin, produced by exposure to 2,4-dinitrophenol, indicated that the angiotensin response of these tissues was more dependent upon energy than the acetylcholine response. The primary action of acetylcholine on smooth muscle cells has been reported to involve an increase in the passive conductance of the membrane to sodium ions (Bolton, 1972) and therefore the energy requirement of an acetylcholine-induced contraction is that of the muscle contraction process itself. Thus, the greater impairment of the angiotensin response that was observed in the experiments with 2,4-dinitrophenol was indicative of the existence of an energy dependent stage in the angiotensin response, distinct from the actual contraction process.

This confirms the results that we reported for guinea-pig ileum (Crocker & Wilson, 1972, 1974) and provides further evidence that the energy dependent stage is involved in the direct action of angiotensin upon smooth muscle. In addition, however, it is evident that the energy supply for this stage of the angiotensin response is variable and dependent upon the tissue and the experimental conditions. In guinea-pig ileum the energy was supplied by aerobic metabolism, but in descending colon and rat uterus, energy may be supplied by aerobic and anaerobic metabolism.

The nature of this energy dependent stage is not yet resolved and further experiments are in progress to investigate a possible relationship between the action of angiotensin and active ion movement.

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