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D. CALCIUM AND THE RELAXANT EFFECT OF ISOPROTERENOL IN THE DEPOLARIZED RAT UTERUS

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Depolarized preparations provide a new way of looking at the action of drugs on smooth muscle. So far as is known, they react to the same drugs as polarized preparations, but their responses are of a simpler pattern, slow and devoid of spontaneous intermittent activity due to local pacemakers and conducted impulses. In some respects potassium-depolarized smooth muscle resembles an isolated actomyosin system, notably in its remarkable sensitivity to calcium, but in contrast to actomyosin it also responds to acetylcholine, adrenaline (E) and other drugs. Depolarized preparations thus provide a means of studying the interrelations between drugs and calcium in the processes leading to contraction and relaxation.

Previous work on calcium and contractile drug responses in depolarized smooth muscle has already been reviewed (20). The present communication summarizes recent work (21) on calcium and the relaxant action of isoproterenol in depolarized smooth muscle.

CONTRACTILE EFFECTS OF CALCIUM IN THE DEPOLARIZED UTERUS

Depolarized muscle responds to calcium by a slow sustained contraction which persists for as long as calcium is present in the solution and subsides when calcium is omitted. This effect may be demonstrated in an isolated horn of rat uterus by recording tension isometrically at 25°C. When the uterus is immersed in calcium-free potassium chloride Ringer solution (KCl Ringer) (150 mM KCl, 5 mM $KHCO_3$, 5 mM glucose) it undergoes a brief contraction followed by relaxation to a new base line. At this stage the muscle is in a partially contracted state: its length under mild tension is less than in sodium-Ringer, and it remains capable of contraction and relaxation in response to drugs.

To elicit a contractile response of the uterus the threshold amount of added calcium is 1 to 2 $\times$ 10^{-6} M calcium chloride (notwithstanding the fact that the bath fluid may contain 10^{-6} to 10^{-5} molar calcium through contamination). The concentrations of calcium which produce a threshold contraction of the depolarized uterus are similar to those known to affect isolated actomyosin from striated muscle; thus Ebashi and Ebashi (7) obtained acceleration of superprecipitation with 5 $\times$ 10^{-6} molar calcium.

Graded concentrations of calcium produce graded contractions of the uterus resulting in an S-shaped log dose-response curve. A 50% contraction is produced by 5 to 20 $\times$ 10^{-5} M calcium. In a few minutes contractile responses reach a horizontal plateau which suggests that an equilibrium between external and free intracellular calcium becomes established.

Effect of chelating agents. When a calcium-chelating agent, ethylenediaminetetraacetic acid (EDTA) or the more specific agent ethylenebis(oxyethyleneni-

trilo)tetraacetic acid (EGTA), is added to KCl Ringer it produces a prompt relaxation of the uterus. When the chelating agent is again omitted, the muscle contracts to the original base line or above. In this way repeated cycles of contraction and relaxation can be induced ostensibly without adding any external calcium. The relaxant effects of chelating agents are graded; a good relaxation can usually be produced with 10^{-4} M EGTA and a minimal effect may be seen with about 4×10^{-6} M EGTA added to the bath.

Mode of action of calcium. It is probable that calcium exerts its contractile effect in depolarized muscle by a direct intracellular activation of the contractile element. Tracer experiments have shown that calcium is taken up rapidly by smooth muscle [taenia coli (1)] but why it should be so active in producing contraction in the depolarized muscle is not known. Possibly sodium antagonizes the contractile action of calcium. Dr. Tkacev, in our laboratory, has found that sodium may relax the depolarized rat uterus contracted by calcium (23; see also 18).

If, as Bozler has suggested (2), calcium is transported by a carrier mechanism into the cell interior, low concentrations may be expected to penetrate readily since carrier transport kinetics resembles diffusion kinetics under low saturation conditions (24) but high concentrations of calcium may be held back (2). Competition for a carrier could account for the competitive antagonism of calcium and magnesium in the depolarized uterus (8). The S-shape of the log dose-response curve indicates a saturation process; this could be saturation of receptors in the contractile element but it could also be saturation of a carrier. The latter would account for the fact that a maximal response to calcium is generally less than the maximal contraction of which the muscle is capable.

The relaxing effect of chelating agents could be explained in two ways. 1) The chelating agent affects an intracellular cycle. It may penetrate into the cell, diminish the concentration of free intracellular calcium and induce relaxation by calcium withdrawal from actomyosin. When the chelate is removed, further calcium might be released from intracellular stores to re-establish a partial contraction. 2) The chelating agent affects an extracellular cycle. A continuous cycle of calcium entry and exit may operate which is disrupted when the concentration of contaminant calcium in the "calcium-free" KCl Ringer is reduced. One of the main difficulties, at this stage, in deciding between alternatives is the uncertainty concerning the function of relaxing factor in uterine smooth muscle (13, 16).

Effect of barium. Barium stimulates depolarized smooth muscle even in the absence of calcium (6, 25). Its contractile response in the depolarized uterus bears a qualitative resemblance to that of calcium and its log dose-response curve is similarly S-shaped. In its contractile effect on the depolarized rat uterus barium is $\frac{1}{5}$ to $\frac{1}{10}$ as active as an equimolar concentration of calcium.

THE ACTIONS OF MAGNESIUM

Magnesium has at least three different actions in the depolarized rat uterus: 1) an irregular contractile effect of its own which exhibits rapid tachyphylaxis; 2) a competitive antagonism of the contractile action of low and medium con-

centrations of calcium (8); and 3) a potentiation of the contractile action of high and maximal concentrations of calcium. Under conditions in which magnesium has no contractile effect on its own it may increase by 25% the maximal tension attainable with calcium.

Glycerol-extracted uterus preparations (3, 14) are contracted by magnesium in the presence of ATP. Calcium may also stimulate contraction, but there is evidence that its contractile effect disappears in well extracted fibres. Briggs (3) has suggested that these differences between the freshly and long extracted glycerinated muscle may be due to the deterioration with time of an attached relaxing factor. Possibly the reactions of the depolarized uterus to calcium and magnesium are due to an interplay between contractile element and relaxing factor which is absent in long-extracted glycerinated preparations.

RELAXANT EFFECTS OF CATECHOLAMINES

As previously shown (9, 10, 19) catecholamines retain their characteristic effects on depolarized isolated smooth muscle preparations immersed in K_2SO_4 or KCl Ringer. Thus E contracts the rabbit uterus and relaxes the rat uterus, whether polarized or depolarized.

Isoproterenol produces a relaxation of the depolarized rat uterus from base line or after it has been stimulated by calcium. In either case it induces a sudden relaxation which is graded according to dose and reversible on washing. The depolarized rat uterus responds initially to 10^{-10} or 10^{-11}, sometimes 10^{-12}, isoproterenol, but its sensitivity declines with time. Noradrenaline (NE) produces similar effects but is about 40 times less active than isoproterenol. The depolarized rat uterus also responds to relaxant drugs which do not belong to the sympathomimetic series, *e.g.*, papaverine.

ANTAGONISM BY DICHLOROISOPROTERENOL (DCI)

The relaxant effects of isoproterenol in the depolarized rat uterus are antagonized by DCI acting in similar concentrations as in nondepolarized muscle. In view of the progressively diminishing sensitivity of the depolarized uterus to isoproterenol, dose ratios (12) were obtained by comparison of two horns of the same uterus, one treated with DCI, the other one untreated.

In the presence of DCI the log dose-response curves of isoproterenol undergo a parallel shift indicating a competitive antagonism. Equilibrium with antagonist is established slowly, *e.g.*, in an experiment with 10^{-6} DCI, dose ratios of 40, 91 and 200 were obtained after 14, 34 and 72 min contact. Similar dose ratios for DCI have been observed in the nondepolarized rabbit intestine (11). It would appear that DCI acts on the same receptors in normal and depolarized muscle, and the same applies to isoproterenol. This is borne out by the high sensitivity of the depolarized uterus to isoproterenol, which is as high as or higher than that of the nondepolarized muscle.

INTERACTIONS OF CALCIUM AND ISOPROTERENOL; MECHANISM OF ACTION OF RELAXANT DRUGS

Insofar as calcium causes contraction and isoproterenol relaxation of the depolarized rat uterus they are functional antagonists. But the two substances

might conceivably interact on a more fundamental level: isoproterenol might produce relaxation by activating a calcium withdrawal mechanism which would tend to reduce the level of free intracellular calcium. The following experiments approach this problem indirectly.

Chelating agents. Chelating agents have a pronounced influence on drug effects in depolarized smooth muscle. A rat uterus horn which has been freshly placed in KCl Ringer (without added calcium) responds to acetylcholine by contraction and to isoproterenol by relaxation and it continues to respond thus for some considerable time although its sensitivity, especially to the relaxant drug, gradually declines. The addition of 10^{-4} EGTA to the bath fluid has the following effects. The preparation relaxes and the responses to both acetylcholine and isoproterenol are greatly reduced or abolished, even if the initial tension is restored by stretching. On replacement with EGTA-free KCl Ringer the muscle contracts and the sensitivity to both drugs is restored more or less completely. Excess calcium induces contraction in EGTA-treated preparations and restores the relaxant effect of isoproterenol. EGTA (10^{-3} M) may abolish irreversibly the relaxant effects of all doses of isoproterenol.

The question arises whether isoproterenol fails to produce relaxation after EGTA simply because it acts on an already relaxed contractile element. This is bound to be part of the explanation but probably not the whole explanation. Thus chelating agents (*e.g.*, high concentrations of EDTA) may produce little relaxation or even contraction but they nevertheless antagonize relaxation by isoproterenol. It can be concluded that chelating agents *per se* antagonize the relaxant action of isoproterenol.

The relaxant effects of papaverine in the depolarized rat uterus are also antagonized by EGTA. This makes it improbable that EGTA inactivates the E receptor; it suggests a more generalized interference with the mechanism of relaxation.

High concentrations of calcium. It might be expected that any mechanism which depends on decreasing free intracellular calcium would be swamped by very high external calcium concentrations. We have therefore tested whether the relaxant effect of isoprenaline is antagonized by high calcium. In experiments in which varying concentrations of calcium were administered followed by constant doses of isoproterenol it was found that approximately equal relaxant effects (in terms of absolute diminution of tension) are produced by isoproterenol at all concentrations of calcium. This result would be difficult to reconcile with the proposed mode of action of isoproterenol unless some barrier for the penetration of high calcium concentrations existed.

Bozler has shown that such a barrier exists for nondepolarized smooth muscle since high outside calcium concentrations failed to reach equilibrium with frog stomach muscle (2) but no such information seems available for depolarized smooth muscle.

LOSS OF SENSITIVITY TO ISOPROTERENOL AND RECOVERY

A feature of the relaxant effect of isoproterenol in the depolarized muscle is its gradual diminution and eventual disappearance. This process can be reversed

by a brief period of immersion in sodium-Ringer. If the muscle is then newly depolarized its sensitivity to the catecholamine is seen to be partly or completely restored.

The following experiment may be cited as an example. A rat uterus suspended in KCl Ringer is stimulated intermittently by calcium and relaxed afterwards by isoproterenol. Initially the muscle is relaxed by 2×10^{-12} isoproterenol but it gradually becomes less sensitive and after 180 min it fails to respond to 2×10^{-6}. At this stage the preparation is immersed in a solution containing equimolar NaCl instead of KCl. When returned to KCl Ringer the muscle undergoes a strong contraction (evidence of depolarization) and is now found responsive to 10^{-8} isoproterenol; after a further 10 min spell in NaCl Ringer followed by another depolarization it responds to 3×10^{-11}. Sodium is not essential for the restoration of sensitivity; the same effect can be obtained by buffered isotonic sucrose. The common factor is presumably repolarization but the point has not yet been investigated directly.

The phenomenon of loss of sensitivity and recovery by brief immersion in Na Ringer is not confined to catecholamines. The relaxant effect of papaverine shows a parallel decline and recovery. To a lesser extent and less consistently the stimulant effect of calcium also declines and recovers. It may be tentatively concluded that a period of repolarization brings about a reorganization of some mechanism responsible for drug effects, particularly relaxant effects.

RELATION BETWEEN RELAXANT EFFECTS OF CATECHOLAMINES IN POLARIZED AND DEPOLARIZED SMOOTH MUSCLE

The extensive work carried out by Bülbring and Kuriyama (4, 5) and Holman (15) on membrane potential and spike activity in smooth muscle has focused attention on these parameters and on the ways in which they are influenced by drugs. E produces hyperpolarization and cessation of spike activity (5), and it seems highly probable that such alterations in the electrical activity of the cell membrane contribute to the relaxant action of E under normal conditions. The question arises as to the role of the drug effects which can be observed in depolarized muscle in a normal context. It could be argued that they are obtained under abnormal conditions and irrelevant to normal ones, but the fact that drugs produce the same effects in polarized and depolarized smooth muscle and act in similar concentrations and on the same receptors suggests that the same processes are activated. The relative weakness of drug responses in the depolarized muscle does not detract from their basic significance since depolarized smooth muscle can hardly be expected to respond normally; its metabolism is profoundly altered (22) and, as shown in the present communication, its machinery for drug responses deteriorates progressively. It can be surmised, however, that electrical events which are intimately bound up with a polarized cell membrane and events which occur independently of membrane polarization both contribute to the normal action of drugs on smooth muscle.

The two types of effect may interact differently according to the particular smooth muscle species and drug investigated. An example of such interaction is provided by a recent investigation by Jenkinson and Morton (17) in the taenia

coli of the guinea-pig. These authors investigated the effects of catecholamines on ionic permeabilities to radioisotopes and correlated them with their mechanical effects. On comparing the relaxant activities of NE and isoproterenol in polarized and depolarized preparations they found that NE was relatively more active in the polarized muscle. This agreed with the finding that NE produced a significant increase in potassium permeability, which in a preparation with normal membrane potential would be expected to induce hyperpolarization and relaxation. By contrast isoproterenol failed to increase potassium permeability, and this suggests that its relaxant action does not depend on this type of effect.

SUMMARY

The potassium-depolarized rat uterus is highly sensitive to calcium. It responds to calcium by contraction and to calcium-chelating agents by relaxation. Magnesium antagonizes or potentiates the contractile effect of calcium in different concentrations.

Isoproterenol produces relaxation which is antagonized competitively by DCI and nonspecifically by chelating agents. The relaxant effect of isoproterenol in potassium-Ringer deteriorates in time but can be restored by temporary immersion in sodium-Ringer. A hypothesis is discussed according to which relaxation is due to a decrease of free intracellular calcium.

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