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The Role of Calcium Ions in the Regulation of Rat Thymocyte Pyruvate Oxidation by Mitogens

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1. Calcium concentrations in the nanomolar range cause a specific stimulation of the oxidation of pyruvate by isolated mitochondria from rat thymus that is sufficient to account precisely for the stimulation of pyruvate oxidation observed when rat thymocytes are incubated with the mitogens concanavalin A or ionophore A23187. 2. Higher concentrations of Ca^{2+} (more than 50nM) inhibit the oxidation of NAD^+-linked substrates by rat thymus mitochondria without affecting the oxidation of succinate or ascorbate+*NNN'N'*-tetramethyl-*p*-phenylenediamine. 3. The addition of Ni^{2+} or Co^{2+} (2mM) to rat thymocytes prevents the response to concanavalin A at the level of pyruvate oxidation without affecting the stimulation of glycolysis induced by this mitogen. In contrast, the complete metabolic response to the ionophore A23187 is abolished by these ions. Ni^{2+} and Co^{2+} interfere with the ability of the ionophore to transport Ca^{2+} across the plasma membrane. 4. Concanavalin A, but not ionophore A23187, increases the respiratory inhibition induced by Ni^{2+} and Co^{2+}. 5. These results support the view that mitogens stimulate lymphocyte pyruvate oxidation through an increase in cellular Ca^{2+} uptake.

A possible role for Ca^{2+} in the regulation of lymphocyte transformation has been widely recognized. Mitogenic plant lectins have been shown to stimulate Ca^{2+} uptake across the plasma membrane (Parker, 1974; Freedman *et al.*, 1975; Averdunk, 1976; Hume & Weidemann, 1978), and their effects on a number of cellular anabolic processes may be mimicked in a Ca^{2+}-dependent manner by the bivalent-cation ionophore A23187 (Luckasen *et al.*, 1974; Maino *et al.*, 1974; Wang *et al.*, 1975; Puckle *et al.*, 1975; Reeves, 1976; Hume & Weidemann, 1978). Yasmeen *et al.* (1977) and Whitesell *et al.* (1977) have demonstrated that the stimulation of glucose transport across the lymphocyte plasma membrane by concanavalin A is dependent on external Ca^{2+}. Stimulation of glucose transport probably accounts for the enhanced aerobic glycolysis observed in mitogen-treated cells [the preceding paper (Hume *et al.*, 1978)], since this reaction is rate-limiting for thymocyte glycolysis (Yasmeen *et al.*, 1977).

An increase in the rate of pyruvate production in mitogen-treated cells as a consequence of stimulated glycolysis is probably not sufficient to account for the specific 30–40% increase in pyruvate oxidation (Hume *et al.*, 1978). We have investigated the alternative possibility that elevated cytoplasmic free Ca^{2+} may be involved in the regulation of mitochondrial pyruvate oxidation by activation of pyruvate dehydrogenase. The results indicate that an increase in free Ca^{2+} in the cytosol in the nanomolar range is sufficient to account precisely for the changes in pyruvate oxidation that are observed in whole cells incubated with concanavalin A or ionophore A23187.

Further evidence for the involvement of Ca^{2+} in the mitogenic stimulation of thymocyte pyruvate oxidation was obtained using the transition-metal ions Co^{2+} and Ni^{2+}. These metals have been shown to interfere with the action of Ca^{2+} in the regulation by insulin of sugar transport in diaphragm muscle (Bihler) 1972, and of pyruvate oxidation in adipose-tissue mitochondria (Severson *et al.*, 1974). In the present paper we report the effects of Ni^{2+} and Co^{2+} on the activation of glycolysis and pyruvate oxidation by concanavalin A and ionophore A23187 in rat thymocytes. The results provide further evidence in support of the view that pyruvate oxidation in lymphoid-tissue mitochondria is controlled by Ca^{2+} independently of tissue pyruvate concentrations. The present work also contrasts the response of lymphocytes to concanavalin A and ionophore A23187 in terms of the sensitivity of the two processes to transition-metal ions.

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Materials and Methods

Materials

Ouabain was obtained from BDH, Poole, Dorset, U.K. [1-^{14}C]Pyruvic acid was obtained from The Radiochemical Centre, Amersham, U.K., and was dissolved in water; portions were stored at −20°C and used within 14 days of receipt. Hyamine hydroxide (a 1M solution in methanol) was from Packard Instruments Co., Downers Grove, IL, U.S.A., and $CaCl_2$ was obtained as a 100mM standard solution from Orion Research, MA, U.S.A. Rubber Suba-Seals (size 33, to fit Beckman glass liquid-scintillation vials) were from Townson and Mercer, Lane Cove, N.S.W., Australia. The sources of all other materials used have been described previously (Suter & Weidemann, 1975; Hume *et al.*, 1978).

Methods

Experiments with isolated mitochondria. Mitochondria were isolated from thymus glands of 7-week-old male Wistar rats as described by Vijayakumar & Weidemann (1976). Mitochondrial respiration was monitored in a Clark-type oxygen electrode (Reed, 1972) in a total volume of 2ml. Free concentrations of Ca^{2+} in the media were calculated as described by Vijayakumar & Weidemann (1976). The assay of mitochondrial [1-^{14}C]pyruvate oxidation was carried out in Beckman glass scintillation vials. Mitochondria were preincubated with the desired concentration of Ca^{2+} for 5min at 25°C and placed on ice. The vials were sealed individually with Suba-Seals, and reactions were initiated by placing each vial in a 37°C shaking water bath and injecting [1-^{14}C]pyruvate through the seal to give a final concentration of 1.0mM (0.1Ci/mol). The reaction was stopped after 5min by injection of citric acid (0.2ml; 65%, w/v). The $^{14}CO_2$ produced was collected by injecting 1.0ml of Hyamine hydroxide into centre wells (1.5ml Eppendorf tubes) and shaking the vials for a further 30min. Finally, saturated $NaHCO_3$ was injected into the medium and the vials were shaken for a further 30min. The centre wells were then removed, wiped, and transferred into 10ml of scintillant (Yasmeen *et al.*, 1977) for counting in a Beckman LS 350 liquid-scintillation counter. Blanks, in which mitochondria were replaced by an equal volume of water, were assayed under identical conditions.

Experiments with isolated thymus lymphocytes. Suspensions of rat thymus lymphocytes were prepared from the thymuses of 6–8-week-old male, outbred Wistar rats (Culvenor & Weidemann, 1976). Cell incubations and subsequent assays of metabolites were carried out as described elsewhere (Suter & Weidemann, 1975). The cell densities used and the concentrations of the agonists investigated are given in the appropriate Table and Figure legends. The initial rate of $^{45}Ca^{2+}$ uptake was measured in thymocytes prepared in the phosphate-buffered saline of Krebs & de Gasquet (1964) modified to contain no Ca^{2+}. The cells (3.3×10^8/ml) were incubated at 37°C in glass vials containing glucose and acetoacetate (both at 5mM), phosphate-buffered saline (with the Ca^{2+} concentration decreased to 0.1mM) and appropriate additions in a total volume of 1.2ml. The low Ca^{2+} concentration is close to saturation for the uptake process (L. M. Russell, D. A. Hume & M. J. Weidemann, unpublished work), and was chosen to avoid excessive dilution of the specific activity. After at least 15min pre-equilibration, reactions were started by simultaneous addition of $^{45}Ca^{2+}$ (approx. 0.1μCi) and concanavalin A, ionophore A23187 or saline. Samples (0.1ml) were removed at 1min intervals, layered on to a bovine serum albumin cushion (1.0ml, 10% bovine serum albumin fraction V in 0.9% NaCl, pH7.4) and centrifuged immediately in an Eppendorf bench centrifuge for 30s. The supernatants were aspirated and the pellets were dissolved in formic acid for radioactivity counting (Yasmeen *et al.*, 1977). Initial rates were derived from the linear portion of the uptake curve.

Results

Calcium effects on mitochondrial pyruvate oxidation

Previous studies in this laboratory have shown that pyruvate dehydrogenase is a low-activity non-equilibrium enzyme in lymphoid tissue (Chan, 1972; Suter, 1973). Ca^{2+} has been implicated in the regulation of this enzyme in a number of other tissues (Denton *et al.*, 1975; Fisher *et al.*, 1973; Schuster & Olson, 1974). Fig. 1 demonstrates that State-3 respiration in the presence of saturating concentrations of pyruvate (3.0mM) plus catalytic amounts of malate (0.5mM) was stimulated in rat thymus mitochondria by very low concentrations of Ca^{2+} (20nM). At higher concentrations (more than 50nM-free Ca^{2+}) the State-3 and State-4 respiration rates became equal and the mitochondria were uncoupled. The maximum stimulation of mitochondrial pyruvate oxidation induced by Ca^{2+} *in vitro* (30–40%) is sufficient to account exactly for the increase in pyruvate oxidation observed in whole cells treated with mitogens. The substrate specificity of the response to 20nM-free Ca^{2+} is shown in Table 1. Succinate supported the fastest rates of State-3 respiration, and the oxidation of this substrate was unaffected by addition of Ca^{2+}. Oxidation of malate, glutamate or citrate was equally unresponsive to Ca^{2+}. However, State-3 respiration in the presence of 2-oxoglutarate was significantly stimulated by 20nM-free Ca^{2+}. The data in Fig. 1 confirm that the increased State-3 respiration in the presence of low Ca^{2+} is accompanied by increased

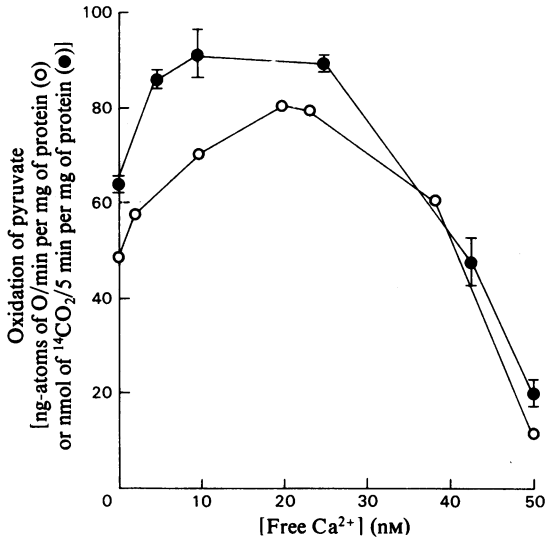

Fig. 1. *Effect of free-Ca^{2+} concentration on the oxidation of pyruvate by isolated rat thymus mitochondria*

The methods used are given in the text. The results are the average of triplicate determinations of O_2 consumption (○) or $^{14}CO_2$ production (●) by rat thymus mitochondria in the presence of 3mM-pyruvate+0.5mM-malate (O_2 consumption) or 1mM-pyruvate+0.1mM-malate ($^{14}CO_2$ production). The error of the determination of O_2 consumption may be seen in Table 1.

Table 1. *Effect of 20nM-free Ca^{2+} on substrate oxidation by rat thymus mitochondria*

Mitochondria were incubated and oxygen consumption was measured by using an oxygen electrode as described under 'Methods'. Substrates were added at a final concentration of 1mM. Results represent the State-3 respiration rate in ng-atoms of oxygen/min per mg of mitochondrial protein±S.E.M. (three experiments) (where appropriate).

Substrates added	Control	+20nM-free Ca^{2+}
Pyruvate (+malate)	62.4±2.4	99.6±6.0
2-Oxoglutarate	75.6±4.8	105.6±6.0
Succinate	123.6	129.6
Malate	57.6	57.6
Citrate	72.0	74.4

pyruvate oxidation. The release of $^{14}CO_2$ from [1-^{14}C]pyruvate (which occurs at the pyruvate dehydrogenase reaction) was stimulated 30–50% by low concentrations of Ca^{2+}. As observed with the oxygen electrode, higher concentrations of Ca^{2+} were inhibitory.

The nature of the inhibitory effect of high Ca^{2+} concentrations is shown in Fig. 2. After addition of 50nM-free Ca^{2+}, thymus mitochondria oxidizing pyruvate failed to respond to addition of ADP or the uncoupler carbonyl cyanide trichloromethoxyphenylhydrazone ('CCCP'). These Ca^{2+}-treated mitochondria also failed to oxidize other NAD^+-linked substrates (3-hydroxybutyrate, citrate; results not shown) but State-3 respiration in the presence of succinate or ascorbate–*NNN'N'*-tetramethyl-*p*-phenylenediamine was unimpaired.

Effects of transition metals on the response to mitogens

At the concentrations used, both Ni^{2+} and Co^{2+} inhibited cellular respiration progressively during a 3h incubation (Fig. 3). These ions also abolished the stimulation of [^{3}H]thymidine incorporation by concanavalin A measured after 48h in culture (D. A. Hume & F. Schweinberger, unpublished work). Both

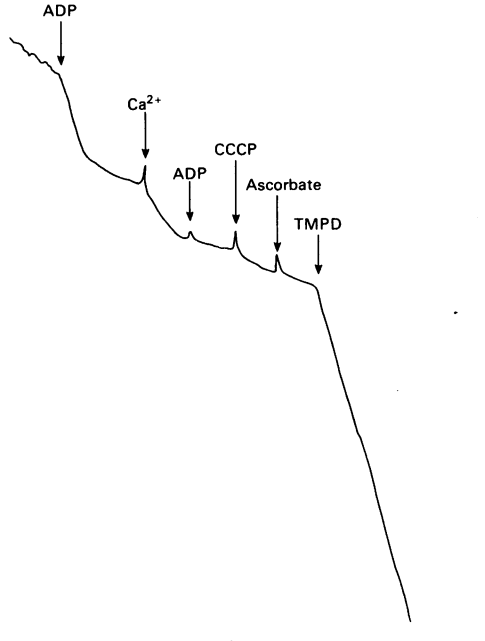

Fig. 2. *Effect of 50nM-free Ca^{2+} on pyruvate oxidation by rat thymus mitochondria*

A typical oxygen-electrode trace obtained in the presence of 3mM-pyruvate+0.5mM-malate is given. Mitochondria (1mg of protein) were suspended in a total volume of 2.0ml, and adenosine bisphosphate (0.1mM), Ca^{2+} (free concentration 50nM, total 0.6mM), CCCP (carbonyl cyanide trichloromethoxyphenylhydrazone) (2μM) and ascorbate + TMPD (*NNN'N'*-tetramethyl-*p*-phenylenediamine) (5mM; 0.05mM) were added at the times indicated by the arrows.

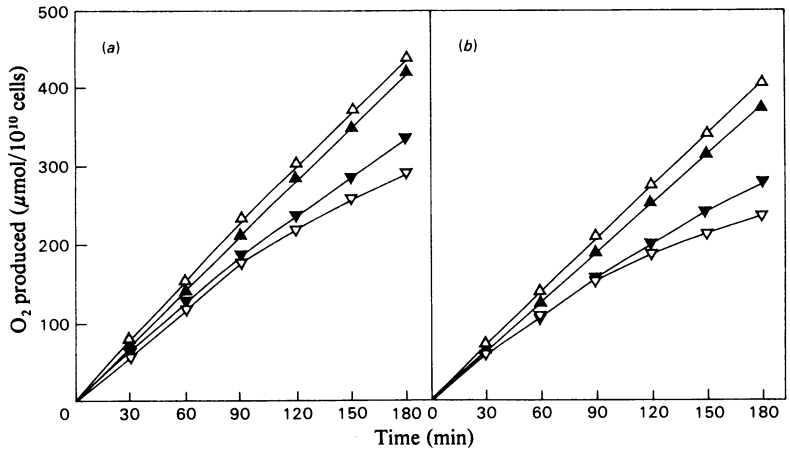

Fig. 3. *Effect of Ni^{2+} and Co^{2+} on O_2 consumption by rat thymocytes*

O_2 consumption was monitored manometrically during the experiments described in Table 2. The points represent five observations in the presence of: ▲, no addition; △, concanavalin A; ▼, Ni^{2+} (*a*) or Co^{2+} (*b*); ▽, Ni^{2+} (*a*) or Co^{2+} (*b*)+concanavalin A.

Table 2. *Effect of Ni^{2+} and Co^{2+} on the response of rat thymus lymphocytes to concanavalin A*

Incubations were performed as described in the text. The final concentrations of added reagents were [U-^{14}C]glucose, 5mM (10^6 c.p.m./flask); concanavalin A (Con A), 50μg/ml; $NiCl_2$ or $CiCl_2$, 2mM. Results are expressed as μmol/h per 10^{10} cells±S.E.M. for five observations on separate cell preparations. *$P<0.05$ for differences from control; **$P<0.05$ for differences from concanavalin A alone when controls are normalized; ***$P<0.01$ for difference from control. *P* values were derived from students *t* test.

	Control	$NiCl_2$	$CoCl_2$	Con A	$NiCl_2$+Con A	$CoCl_2$+Con A
O_2 consumption	426±25	336±20	279±13	441±29	300±20	248±12
CO_2 production	409±24	342±31	275±20	454±36	285±19	241±14
10^{-3}× Total radioactivity in $^{14}CO_2$ (c.p.m.)	48.5±4.4	34.9±3.4	28.3±1.5	70.4±7.8	39.3±2.7	25.3±21
10^{-3}× Sp. radioactivity of $^{14}CO_2$ (c.p.m./μmol)	3.35±0.3	3.11±0.37	2.84±0.17	4.68±0.51	4.11±0.24**	2.61±0.06
Glucose disappearance	69.6±9.8	51.1±10.8	—	148.5±6.9***	148.0±11.0***	—
Lactate production	39.1±6.8	32.5±10.5	53.1±7.8	152.0±12.2***	187.0±11.8***	135.4±19

Table 3. *Effect of Ni^{2+} and Co^{2+} on the response of rat thymus lymphocytes to ionophore A23187*

Incubations were carried out as described in the text. Ionophore A23187 was used at a final concentration of 0.4μg/ml, and other reagents were used at the concentrations given in Table 2. Results are expressed as μmol/3 h per 10^{10} cells+S.E.M. (where appropriate) of three observations on separate cell preparations. *$P<0.05$ for difference from control; **$P<0.02$ for difference from control.

	Control	+$NiCl_2$	+$CoCl_2$	+A23187	+$NiCl_2$+A23187	+$CoCl_2$+A23187
O_2 consumption	416	325	318	424	328	350
CO_2 production	424	338	330	440	316	335
10^{-3}× Radioactivity in $^{14}CO_2$ (c.p.m.)	49.7±2.8	34.2±0.8	28.8±1.5	65.9±6.2*	29.1±2.2	28.5±2.9
10^{-3}× Sp. radioactivity of $^{14}CO_2$ (c.p.m./μmol)	3.36±0.13	2.95±0.12	2.60±0.08	4.27±0.29*	2.67±0.24	2.71±0.37
Glucose disappearance	59.8±7.1	40.6±11.8	—	100.3±25**	38.9±13.8	—
Lactate production	44.4±4.5	27.5±4.5	56.1±3.2	131.4±21.1**	28.2±3.2	59.9±11.3

concanavalin A and ionophore A23187 caused a small, but highly reproducible, stimulation of respiration in thymocytes when parallel incubations were compared (Fig. 3 and Table 3). However, concanavalin A (Fig. 3) but not ionophore A23187 (Table 3) inhibited respiration in the presence of Ni^{2+} and Co^{2+} and the percentage inhibition increased with time.

As previously reported (Hume *et al.*, 1978), the addition of concanavalin A to thymocytes caused a doubling in the rate of glucose utilization, the major fate of which was conversion into lactate. In addition, concanavalin A specifically stimulated the flow of glucose carbon to CO_2 (Table 2). In control incubations with [U-^{14}C]glucose, both Ni^{2+} and Co^{2+} substantially decreased the appearance of label in CO_2. This inhibition was proportional to the inhibition of cellular respiration and was not accompanied by a decrease in the specific activity of the CO_2 produced (Table 2). Concanavalin A increased both the net amount and the specific radioactivity of the $^{14}CO_2$ produced. In the presence of Ni^{2+} or Co^{2+}, neither of these responses to concanavalin A was observed (Table 2). In contrast with the inhibitory effect of Ni^{2+} and Co^{2+} on concanavalin A-stimulated pyruvate oxidation, neither ion appeared to have a selective effect on concanavalin A-stimulated glucose uptake or lactate accumulation (Table 2).

The stimulation of glucose oxidation in thymocytes by ionophore A23187 exactly paralleled that observed in response to concanavalin A (Table 3). The two responses differed, however, in their sensitivity to Ni^{2+} and Co^{2+}. Both metal ions completely abolished the stimulation of glucose uptake, lactate production and pyruvate oxidation observed in response to ionophore A23187 (Table 3). We therefore investigated the possibility that Ni^{2+} and Co^{2+} might be acting by interfering directly with the ionophoric properties of ionophore A23187. Table 4 suggests that this might be the case. Both Ni^{2+} and Co^{2+} stimulated the initial rate of cellular $^{45}Ca^{2+}$ uptake slightly when no other stimulating agent was present, but had no significant effect on the stimulation of $^{45}Ca^{2+}$ uptake by concanavalin A (Table 4). Ionophore A23187 caused a much greater stimulation of $^{45}Ca^{2+}$ uptake than concanavalin A, but the ionophore response, in contrast, was eliminated almost completely by both Ni^{2+} and Co^{2+}.

Table 4. *Effects of transition metals on $^{45}Ca^{2+}$ uptake in rat thymocytes*

Experiments were performed as described in the text. The initial rate of transport of $^{45}Ca^{2+}$ is given as nmol of $^{45}Ca^{2+}$ accumulated/min per 10^{10} cells +S.E.M. of three observations, in duplicate, on separate cell preparations. Ni^{2+} and Co^{2+} were added at a concentration of 2mM.

	Control	+Concanavalin A	+Ionophore A23187
Control	3.5±0.4	19.8±2.8	71.5±15.4
Ni^{2+}	4.9±1.1	19.1±1.2	13.1±5.0
Co^{2+}	5.4±0.8	Not tested	6.5±0.5

Discussion

The activity of mitochondrial pyruvate dehydrogenase is believed to be controlled by the interconversion of active (dephosphorylated) and inactive (phosphorylated) forms of the enzyme (Denton *et al.*, 1975; Linn *et al.*, 1969*a*,*b*). Studies on pyruvate dehydrogenase phosphate phosphatase have revealed that Ca^{2+} may lower the K_m of the enzyme for its substrate, leading indirectly to the activation of pyruvate dehydrogenase (Denton *et al.*, 1975). The response of pyruvate oxidation to added Ca^{2+} has been observed in isolated mitochondria from a number of other tissues (Schuster & Olson, 1974; Fisher *et al.*, 1973), but there has been no indication of the sensitivity of this process to low free Ca^{2+} in a Ca^{2+}-buffered system. The inhibitory effects of Ca^{2+} at concentrations above 50nM on the oxidation of NAD-linked substrates is very similar to that exerted by rotenone, and may be related to the formation of Ca–NADH complexes within the mitochondrial matrix (Chance, 1965). More importantly, from a physiological viewpoint, the results suggest an upper limit of 50nM for the free Ca^{2+} concentration in the cytosol, above which there is irreversible damage to mitochondrial function. The results are consistent with the proposal that concanavalin A and ionophore A23187 activate cellular pyruvate oxidation by increasing the concentration of free Ca^{2+} in the cytosol. The data in Table 4 suggest that the selective permeability of the plasma membrane to Ca^{2+} is relatively unimpaired by Ni^{2+} and Co^{2+}. Similarly, since neither ion interferes with the ability of concanavalin A to stimulate glycolysis, we consider that the specific inhibitory effect of Ni^{2+} and Co^{2+} on concanavalin A-stimulated pyruvate oxidation constitutes further evidence that the mitogen effect at this level is independent of the stimulation of glycolysis. The most likely site of action of Ni^{2+} and Co^{2+} would appear to be pyruvate dehydrogenase phosphate phosphatase. Severson *et al.* (1974) have shown that activation of this enzyme by Ca^{2+} is blocked by Ni^{2+}.

The ability of concanavalin A to intensify the inhibition of respiration exerted by Ni^{2+} and Co^{2+} suggests that the lectin enhances the access of these ions to the mitochondria, which may be taken as further evidence for an increase in plasma-membrane permeability or 'leak flux' in mitogen-treated cells (Averdunk, 1976). We cannot, at present, clarify the

mechanism whereby the transition metals block cellular Ca^{2+} uptake, and the subsequent metabolic response, in the presence of the ionophore. It seems unlikely that the ionophore actually transports Ni^{2+} or Co^{2+}, since ionophore A23187 does not amplify the transition-metal-ion-induced respiratory inhibition. The effects of Ni^{2+} and Co^{2+} are not mimicked by Mn^{2+} (Resch & Bouillon, 1978), which has a 100-fold higher affinity than Ca^{2+} for the ionophore (Reed & Lardy, 1972). Nevertheless, evidence is presented that the metabolic response to the ionophore is dependent on its ability to transport Ca^{2+}, which is at variance with the conclusion reached by Resch & Bouillon (1978) and Hesketh *et al.* (1977).

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