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Vanadate-Activated Calcium Influx in A431 Cells Is Dependent on the Plasma Membrane Potential

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Vanadate can activate the uptake of Ca in A431 epidermal carcinoma cells by two- to fivefold with no detectable lag period. Preincubation with epidermal growth factor (EGF) to down-regulate the EGF receptor prevents subsequent stimulation by EGF but not that by vanadate. Ca uptake is sodium-independent and is not activated by depolarization in high KCl. On the contrary, vanadate-stimulated uptake is completely inhibited by decreasing the plasma membrane potential from about −65 to −30 mV. These results demonstrate that the EGF receptor is not itself functioning as a Ca channel, that vanadate is not acting at the level of EGF receptor, and that the Ca transport system exhibits an unusual potential sensitivity in that it is inhibited by depolarization of the plasma membrane.

Key words: epidermal growth factor, depolarization, epidermal carcinoma cells, vanadate, calcium influx, plasma membrane potential A431 cells

Unlike many other mitogens, epidermal growth factor (EGF) and vanadate both appear to induce rapid increases in cytosolic Ca^{2+} by raising the plasma membrane permeability to Ca^{2+} [1,2] rather than by triggering the release of intracellular Ca^{2+} stores [3]. Neither the coupling mechanism between these mitogens and the change in Ca^{2+} permeability nor the mechanism of Ca^{2+} transport are yet understood. Vanadate is a potent inhibitor of phosphotyrosyl phosphatases [4] and significantly increases the level of autophosphorylation of EGF receptors in A431 cell membrane preparations [5]. Vanadate has also been shown to mimic the effect of EGF on the Na^+/H^+ exchange system of A431 cells [6]. However, it was not clear from these studies whether the effects of vanadate are mediated via the EGF receptor. We therefore examined the effect of down-regulation of the receptor induced by preincubation with EGF and found that preincubation with EGF blocked the subsequent stimulation of Ca^{2+} influx by EGF but not that by vanadate. It also did not prevent inhibition of the vanadate stimulation by phorbol esters. Pretreatment with vanadate

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had no effect on the activation of Ca^{2+} uptake by either agent [7]. These results suggest that vanadate acts at a site distinct from or distal to the EGF receptor. In an initial characterization of the mitogen-responsive Ca^{2+} transport system we observed that both EGF and vanadate stimulate the initial rate of uptake of Ca^{2+} into A431 cells by two- to fivefold [7]. No detectable lag period precedes the stimulation by either agent, and the vanadate stimulation appears to be transient. Stimulation by vanadate and EGF is not additive, indicating that both agents act on the same Ca^{2+} transport system [7].

We also observed that raising the extracellular K^+ concentration inhibited the vanadate-activated Ca^{2+} influx [7]. We have now characterized this response in more detail and demonstrated that the transport system is dependent on the plasma membrane potential.

The apparent K_m and V_{max} for the basal influx of Ca^{2+} by the cells were determined to be 83 μM and 0.26 fmol/cell/min, respectively, and only the V_{max} was significantly altered on addition of vanadate (data not shown). Assuming that the unidirectional influx, measured using $^{45}Ca^{2+}$ as tracer, represents only a net flux of Ca^{2+}, this initial rate could carry a maximum inward current of about 0.8 pA (basal rate of influx).

The best-characterized Ca^{2+} transport systems that might increase Ca^{2+} influx into a cell are the voltage-activated Ca^{2+} channel [8] and the $3Na^+/Ca^{2+}$ exchange system [9]. To determine whether either of these systems is activated in A431 cells, we examined the effects of depolarization in high KCl and of altering extracellular or intracellular Na^+. Since $3Na^+/Ca^{2+}$ exchange is electrogenic, depolarization might be expected to increase Ca^{2+} influx via this system. The channel is also activated by depolarization. However, as can be seen from Table I, 40 mM KCl completely blocked the stimulatory effect of vanadate, even reducing uptake to below the basal rate. Moolenar et al [10] have demonstrated that the plasma membrane potential in A431 cells is dominated by the K^+ permeability, and we calculate that 40 mM K^+ would cause a fall in the potential from about -68 mV to -33 mV. The Ca^{2+} influx was not dependent on extracellular Na^+, since partial replacement of the NaCl by choline chloride had no effect (Table I). Moreover, the influx was not being inhibited simply by competition with high extracellular K^+, since depolarization by preincubation with ouabain in low KCl medium also inhibited uptake. Again, the inhibition decreased uptake to well below the basal rate of influx. Since treatment with ouabain would also increase intracellular Na^+, which would increase Ca^{2+} influx via a $3Na^+/$

TABLE I. Effects of Na^+ and Depolarization on Vanadate-Stimulated Ca^{2+} Influx in A431 Cells

Preincubation	Treatment (200 μM)	Initial rate of $^{45}Ca^{2+}$ influx (% of control)
—	Vanadate	225 ± 23.4
40 mM KCl	Vanadate	72 ± 5.1
50 mM choline Cl	Vanadate	294 ± 39.3
5 μM valinomycin	Vanadate	77 ± 29.7
100 μM ouabain[a]	Vanadate	58 ± 5.5

[a]A431 cells were provided by S. Cohen (Vanderbilt, TN) and cultured as described previously [7]. Cells were pretreated with ouabain for 30 min at 37°C prior to initiation of $^{45}Ca^{2+}$ influx. All other preincubations were for 3–5 min only. Influx was measured as described previously [7], using confluent monolayers in 96-well plates incubated in buffered saline with 100 μM $CaCl_2$ at 37°C.

Ca^{2+} exchange, it appears very unlikely that such an exchange system mediates a significant Ca^{2+} flux in A431 cells. Valinomycin also inhibited the vanadate-stimulate uptake of Ca^{2+} (Table I), an effect similar to that reported in leukemic T cells for the T cell receptor-associated Ca^{2+} influx [11]. The mechanism of inhibition is not clear. It is unlikely to be due to hyperpolarization, as has been suggested for T cells [11], since the potential in A431 cells is already controlled by K^+ [10]. Valinomycin might conceivably be causing a rapid depolarization or inhibiting the Ca^{2+} transport system directly.

To characterize the effects of depolarization in more detail, vanadate-stimulated Ca^{2+} uptake was measured over a range of external KCl concentrations and expressed as a function of the membrane potential, calculated from the Goldman equation [10]. The results (Fig. 1) indicate that the inhibitory effect of depolarization is half-maximal at about −55 mV (interior negative).

We have found that both EGF and vanadate can stimulate Ca^{2+} influx into A431 cells via the same transport system, but through distinct mechanisms. The properties of the transport system are distinct from those expected of a $3Na^+/Ca^{2+}$ exchange system or a voltage-activated Ca^{2+} channel. The mode of Ca^{2+} influx remains obscure. It is, however, reminiscent of the IgE-activated Ca^{2+} channel found in mast cells [12] and of the Ca^{2+} transport system associated with the T3-T cell receptor in leukemic T cells [11]. Definitive evidence that the system represents a novel form of voltage-sensitive Ca^{2+} channel might prove difficult to obtain, since the calculated conductance per cell will be less than 4 pA even under fully activated conditions.

The mechanism by which vanadate activates the Ca^{2+} flux is unclear, particularly since no lag is detected before the onset of stimulation, and EGF receptors do

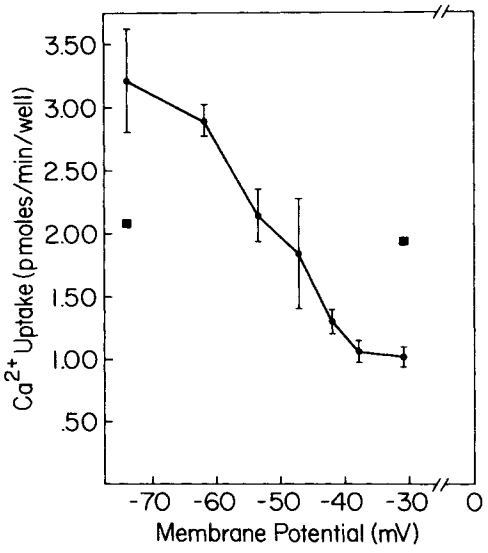

Fig. 1. Effect of membrane potential on vanadate-stimulated Ca^{2+} influx (●). The plasma membrane potential was altered by partial replacement of NaCl with KCl in the buffered saline used for the uptake assays. Absolute potentials were calculated from the Goldman equation given by Moolenar et al [10]. Initial rates of Ca^{2+} influx were measured using $^{45}Ca^{2+}$ as tracer, as described in Table I. Error bars are ± 1 SD (N = 3). Basal influx was measured at two points (■).

not appear to be required. It is possible that the agent is acting on a distinct receptor system and that entry into the cell is not required. Since vanadate has been widely used as an inhibitor of phosphotyrosyl phosphatases to study the action of oncogenes and growth factors [eg, 4,5,13] it will be of some importance to determine with what other systems it can interact.

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