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Evidence for the involvement of carboxyl groups in passive calcium uptake by liver plasma membrane vesicles and in agonist-induced calcium uptake by hepatocytes

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The hydrophobic reagents DCCD and EEDQ, each of which reacts with protein carboxyl groups, were found to inhibit both passive Ca^{2+} uptake by plasma membrane vesicles isolated from rat liver and agonist-induced Ca^{2+} uptake by hepatocytes. The data raise the possibility that the Ca^{2+} inflow pathway(s) in liver has a specific requirement for a reactive carboxyl group or groups.

Calcium; Hepatocyte; Channel; Carbodiimide

1. INTRODUCTION

Stimulation of cellular Ca^{2+} uptake in liver is a major response consequent to the action of α-adrenergic agonists (for a recent review see [1]). The chemical nature of the stimuli that induce such uptake is still largely unknown, although there are suggestions that inositol 1,3,4,5-tetraphosphate [2,3] and/or GTP [4] might be involved. Similarly, the route(s) by which the ion traverses the plasma membrane in gaining access to the cell cytoplasm is largely unknown.

In efforts to characterise, in molecular terms, the Ca^{2+} uptake route in liver, we described initially a passive Ca^{2+} uptake process in plasma membrane vesicles obtained from rat liver that occurs independently of ATP hydrolysis and which has properties typical of a protein-catalysed process [5]. Since we have been unable so far to hormonally stimulate this uptake by isolated vesicles [5] it has yet to be determined if this pathway is the same as that involved in agonist-induced Ca^{2+} inflow as observed in hepatocytes and in perfused rat liver. This paper describes the next stage in this characterisation, namely that of ascertaining whether specific group reative reagents, capable of interacting with carboxyl groups on proteins, inhibit Ca^{2+} uptake in these vesicles and in hepatocytes.

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Abbreviations: CMCD, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide; DCCD, *N,N'*-dicyclohexylcarbodiimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline

2. EXPERIMENTAL

Plasma membrane vesicles were isolated from male Wistar rats of approx. 300 g body weight exactly as described previously [6]. Hepatocytes were prepared by a modification of the methods described by Berry and Friend [7] and Barritt et al. [8] as indicated in Altin and Bygrave [9] except that the final wash was carried out with complete Eagle's minimal essential medium containing 600 μM Ca^{2+} and 10 mM Hepes buffer (pH 7.4) without phenol red. The cells were resuspended in this medium (10 mg cells wet weight/ml), dispensed in 1 ml portions and incubated for 20 min at 37°C before the addition of any agents. Cell viability, as judged by the exclusion of Trypan blue, always exceeded 90%.

Passive influx of $^{45}Ca^{2+}$ by freshly isolated membrane vesicles was determined by membrane filtration as described by Bygrave et al. [5].

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Influx of $^{45}Ca^{2+}$ into hepatocytes was carried out as described by Biden et al. [3]. Other details are included in the legends to the figures. All experiments were carried out with constant shaking and gassing with O_2/CO_2 (19:1).

Data shown are from one of at least 3 experiments that gave similar results.

3. RESULTS AND DISCUSSION

Information about the molecular nature of the protein(s) involved in Ca^{2+} uptake/influx by cells is confined largely to those proteins that have been isolated from 'excitable' cells. The molecular characterisation of these calcium channels has been aided considerably by the discovery and application of calcium antagonists that block Ca^{2+} entry into cells of several tissues like the myocardium, the brain, the smooth muscle cells of the gut and vasculature and skeletal muscle. One such antagonist, 1,4-dihydropyridine (nifedipine), has proved particularly useful in that as a radioligand, it has provided a means to isolate and characterise the Ca^{2+} channel in several tissues (for reviews see [10,11]). The apparent lack of inhibition by these calcium channel blockers of Ca^{2+} inflow in liver unfortunately prevents this approach to be adopted in that tissue.

Some time ago Williams [12] pointed out that the principal groups to which Ca^{2+} binds are oxyanions such as carboxylates and phosphates and that in model complexes the binding strength of Ca^{2+} can be shown to be related to the number of carboxylates. With this knowledge in mind, and in an extension of our interest in the mechanisms of Ca^{2+} inflow into liver cells (for a review see e.g. Altin

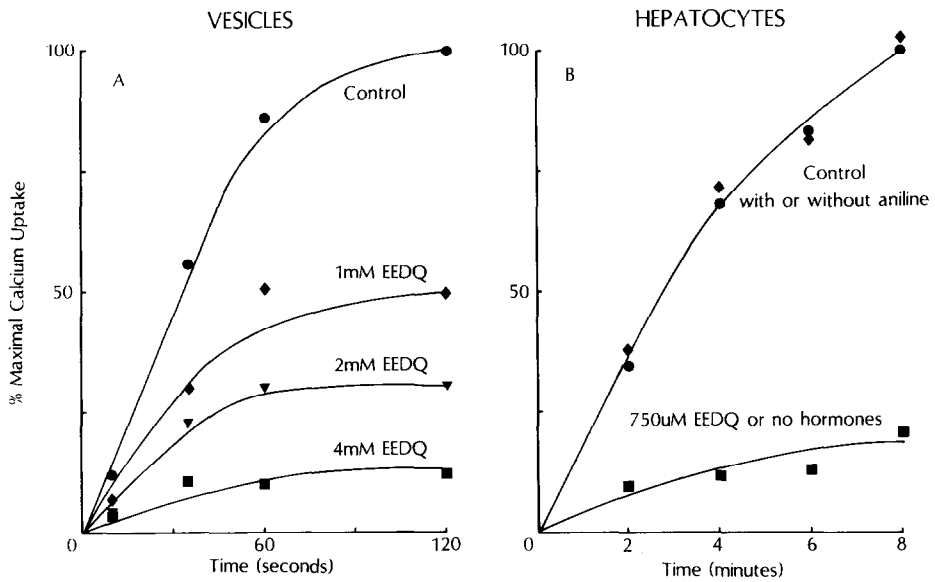

Fig.1. Inhibition by EEDQ of passive Ca^{2+} uptake by plasma membrane vesicles and of agonist-induced Ca^{2+} uptake by hepatocytes. Passive Ca^{2+} uptake (A) was measured in an incubation medium consisting of 120 mM NaCl, 12 mM Hepes buffer and 5 mM KCl in 500 μl final volume at 37°C, pH 7.4. EEDQ, dissolved in DMSO, was added as indicated and the control contained an equal concentration of DMSO alone; the final DMSO concentration was 1%. Membrane vesicles (250 μg protein per reaction medium) were preincubated for 15 min with the added agents prior to the addition of radiolabelled Ca^{2+} containing 100 μM $^{40}Ca^{2+}$. Samples were then taken at the times indicated, filtered through Sartorious membrane filters (0.2 μM pore size), immediately washed with 2.0 ml of incubation medium containing 1 mM EGTA to remove non-sequestered $^{45}Ca^{2+}$, the filters dried and counted for radioactivity as described in [5]. Agonist-induced Ca^{2+} uptake by hepatocytes (B) was measured in an incubation medium consisting of Krebs-Henseleit bicarbonate buffer (pH 7.4) containing physiological amino acids, 5 mM glucose and 0.5% defatted bovine serum albumin in a final volume of 1 ml; all experiments were carried out at 37°C with constant shaking and gassing with O_2/CO_2 (19:1). Following a 20-min preincubation period, EEDQ with or without aniline or DMSO alone in the case of the control, were added. One min later 100 nM glucagon or 200 μM cAMP was added and a further 1 min later 100 nM vasopressin. 30 s after the addition of the final hormone, tracer $^{45}Ca^{2+}$ (0.63 μCi) was added, 100-μl samples then taken at the times indicated and washed on Millipore membrane filters (5 μm pore size) with approx. 4 ml of a cold solution containing 135 mM NaCl, 12 mM Hepes, 2 mM EGTA (pH 7.4). The filters were then dissolved and counted as described in [3]. Maximal Ca^{2+} uptake (i.e. 100%) for (A) was 1.5 nmol/mg protein, and for (B) 2 nmol/mg cells wet wt.

and Bygrave [1]), we examined the effect of several *N,N'*-disubstituted carbodiimides (see e.g. [13]) and of an *N*-carbethoxydihydroquinoline [14], compounds that have been used widely as protein carboxyl groups modifying reagents (see e.g. [15]). This examination has been carried out on Ca^{2+} uptake by vesicles derived from the plasma membrane of rat liver [5] and by hepatocytes in the presence of Ca^{2+}-mobilising agonists.

We showed recently that plasma membrane vesicles of rightside-out orientation isolated from rat liver are able to take up Ca^{2+} independently of a requirement for ATP and have argued that such uptake could reflect one of the pathways for Ca^{2+} inflow into liver cells [5]. Data in fig.1A show that such passive Ca^{2+} uptake is inhibited by EEDQ; approx. 50% inhibition of the initial rate of uptake is obtained with 1 mM EEDQ. Data in fig.1B show that Ca^{2+} uptake into hepatocytes stimulated by the synergistic action of glucagon and vasopressin (reviewed in [1]) is inhibited similarly by EEDQ; 750 μM EEDQ reduces the initial rate of uptake to a value comparable to that observed in the absence of the Ca^{2+}-mobilising agonists.

Having found that EEDQ has such inhibitory effects, we then tested the effects of three different carbodiimides. Data in fig.2A show that the hydrophobic DCCD also inhibits passive Ca^{2+} uptake by the plasma membrane vesicles but that by contrast with DCCD and EEDQ (fig.1A), both the hydrophilic EDAC and the hydrophilic CMDC even at concentrations of 5 mM, are not so effective as inhibitors. The data in fig.2B show that Ca^{2+} uptake into hepatocytes also supported by the synergistic action of glucagon and vasopressin, is very sensitive to the action of DCCD; 100 μM DCCD for example almost completely inhibits such Ca^{2+} uptake. In these latter experiments the effects of EDAC and CMCD were not assessed.

Care was taken to assess whether in the case of hepatocytes, the carboxyl group reactants were acting on Ca^{2+} inflow and not on the ability of the hormones to stimulate Ca^{2+} uptake through for example their second messenger action. To examine this, we tested first whether DCCD and EEDQ would inhibit the ability of glucagon and vasopressin to stimulate glucose output. In experiments not shown here, we observed that these compounds had no effect on agonist-stimulated glucose output in otherwise identical experimental conditions. If anything, glucose output was stimulated by the presence of DCCD. Second, we were able to show

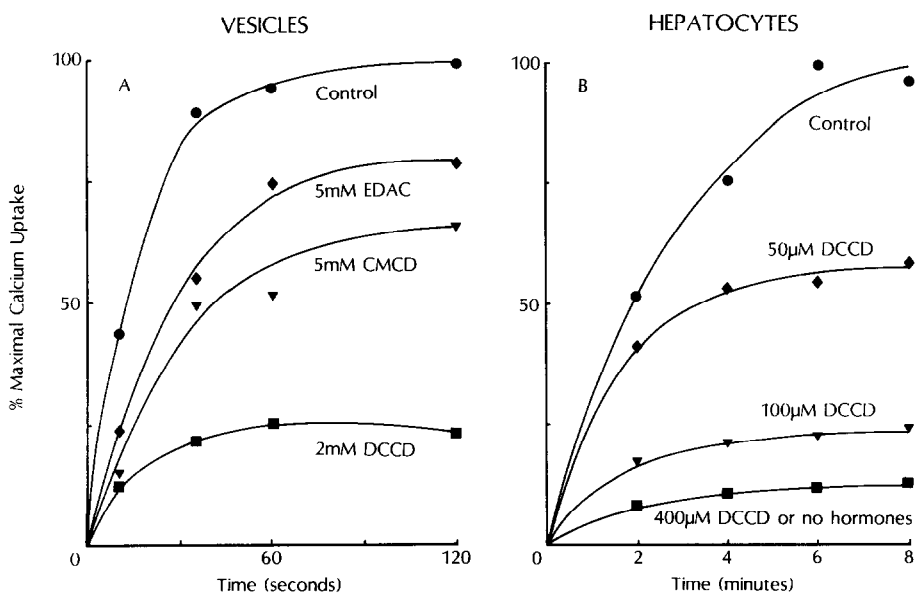

Fig.2. Inhibition by DCCD of passive Ca^{2+} uptake by plasma membrane vesicles and of agonist-induced Ca^{2+} uptake by hepatocytes. Each set of experiments was carried out exactly as described in fig.1 except that DCCD or EDAC or CMCD was present at the concentrations indicated. The values obtained for the controls in each case were similar to those indicated in fig.1.

that when glucagon was replaced by cyclic AMP as inducer of the synergistic action on Ca^{2+} inflow (see [1]), or when vasopressin was replaced by either ATP or phenylephrine as Ca^{2+}-mobilising agonist (see [16]), DCCD and EEDQ were still effective inhibitors of Ca^{2+} inflow by hepatocytes. Results of these experiments thus led us to conclude that DCCD and EEDQ were having direct effects on the Ca^{2+} inflow protein/channel and not on either the receptors for the agonists located on the outer surface of the plasma membrane, or on any relevant site of action of any second messenger generated therefrom.

4. CONCLUDING COMMENTS

These studies extend our investigation into the molecular basis of Ca^{2+} inflow across the plasma membrane in liver cells and vesicles by providing evidence that the inflow pathway(s) appears to have a specific requirement for a reactive carboxyl group or groups. The finding that the hydrophobic carboxyl group reactants are more potent than the water-soluble carboxyl group reactants in their inhibitory action on Ca^{2+} inflow, suggests that this could be an important and useful tool with which to study further the molecular nature of the Ca^{2+} inflow protein(s) in liver cells particularly in aiding the discrimination of 'relevant' from 'non-relevant' carboxyl groups on the external surface of the liver plasma membrane.

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