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On the Mechanism of Action of Econazole, the Capacitative Calcium Inflow Blocker

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The ability of bovine serum albumin to reverse the inhibitory action of econazole and the unsaturated fatty acid oleate on store-dependent Ca^{2+} inflow was examined in Ehrlich ascites tumour cells. We report that inhibition of Ca^{2+} inflow by both compounds is reversed immediately upon addition of bovine serum albumin. It is concluded that the inhibitory action of econazole resembles that of unsaturated fatty acids. The mechanism appears to be one pertaining to non-specific events at the plasma membrane, possibly involving alterations in plasma membrane fluidity/structure.

Movement of Ca^{2+} across cell and subcellular membranes is an obligatory event in many physiological responses in all cells and tissues (see eg. [1]). Elucidation of the mechanisms by which these movements occur is essential for understanding not only the nature of the molecular events involved, but also for investigating the molecular basis of diseases in which aberrant Ca^{2+} movement occurs. A variety of pharmacological agents have been employed to investigate mechanisms of cellular Ca^{2+} fluxes in excitable tissues (see eg. [2]). Others, especially substituted imidazole compounds (see eg. [3]) have been described which appear to specifically inhibit capacitative Ca^{2+} inflow into cells of non-excitable tissues. However the mechanisms involved in such inhibition remain unclear. These compounds are of additional interest in that they have the potential to provide information about the channel(s) responsible for capacitative Ca^{2+} inflow—information about which there currently is widespread interest.

In a recent study [4] we reported that econazole, a prototype of one of these inhibitors [5] mobilized intracellular Ca^{2+} in Ehrlich ascites tumour cells and that this Ca^{2+}-mobilizing action was reversed by bovine serum albumin. This led to the consideration that this action of econazole occurred via a non-specific interaction, perhaps on the plasma membrane [4]. Moreover, we have also shown [6] that unsaturated fatty acids inhibit capacitative Ca^{2+} inflow, an event also reversed by bovine serum albumin. In light of this we compared the ability of bovine serum albumin to reverse the inhibitory action of econazole and oleic acid on capacitative Ca^{2+} inflow in Ehrlich cells.

MATERIALS AND METHODS

Cells

Ehrlich ascites tumour cells (hyperdiploid strain) were transplanted weekly in the peritoneal cavity of 25-30g male Swiss albino mice and harvested 6-9 days after transplantation [7].

[Ca^{2+}]i Measurements

Cells were loaded with 3 μM fura-2 acetoxymethyl ester according to standard procedures. After loading, the cells were kept at room temperature in a medium containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose and 25 mM Hepes (pH 7.4) until used. Immediately before the experiment, 1 ml of the cell suspension was rapidly centrifuged, and resuspended in fresh medium containing no added Ca^{2+} and 0.2 mM EGTA, to give a final concentration of 2.5×10^6 cells/ml. Fluorescence was measured with a Perkin-Elmer LS3B fluorimeter (excitation and emission wavelengths, 340 and 509 nm, respectively) equipped with magnetic stirring and temperature control. At the end of each incubation, digitonin (50 μg/ml) and EGTA (20 mM) were added in order to measure maximal (Fmax) and minimal (Fmin) fluorescence values, respectively. Fluorescence mV output signals were acquired at 0.25 or 0.5 sec intervals, using a MacLabTM hardware (AD Instruments), equipped with a Chart v3,2.5. software. The Kd for the Ca^{2+}-fura-2 complex was assumed to be 185 at 30°C [8]. Values of [Ca^{2+}]i were calculated by using CA Cricket Graph III software according to the formula $[Ca^{2+}]i = Kd[(F-Fmin)/(Fmax-F)]$.

Materials

Fura 2-AM was from Molecular Probes, Eugene, OR, USA. Thapsigargin, econazole, oleic acid, were from Sigma, St. Louis, MO, USA. Fatty acid-free bovine serum albumin was from Boehringer Mannheim, Germany. All other chemicals were of analytical grade.

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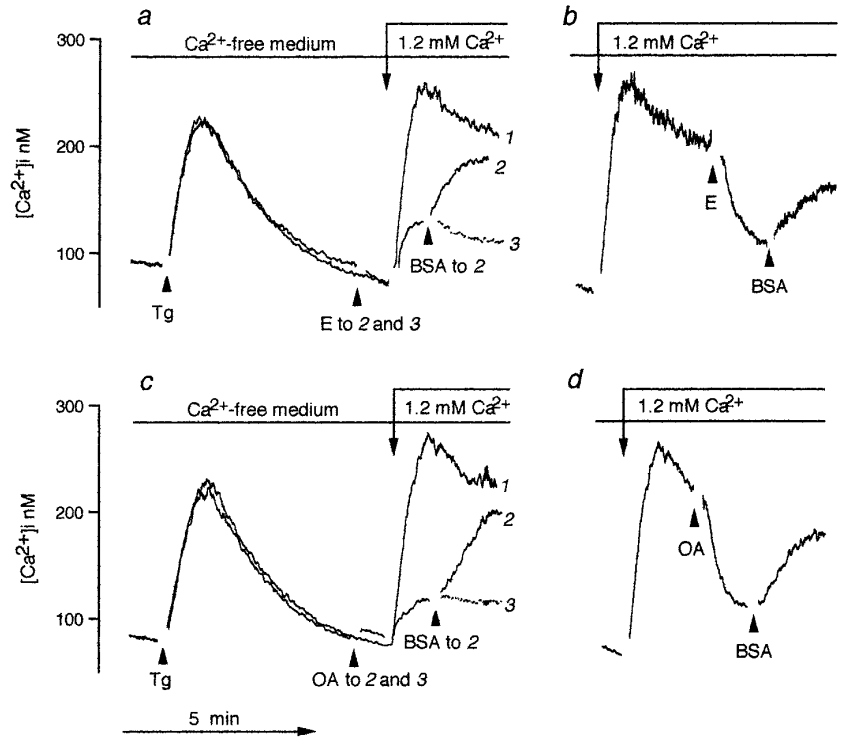

FIG. 1. Econazole inhibits thapsigargin-induced capacitative Ca^{2+} inflow by Ehrlich ascites tumour cells in an albumin-reversible manner. Cells were loaded with Fura-2 and resuspended in Ca^{2+}-free medium (no Ca^{2+}, plus 0.2 mM EGTA) to give 1.25×10^6 cells/ml, as detailed in the "*Materials and Methods*" section. Additions as indicated were thapsigargin (Tg, 0.5 μM), econazole (E, 15 μM), oleic acid (OA, 5 μM), $CaCl_2$ (Ca^{2+}, 1.2 mM), and fatty acid-free bovine serum albumin (BSA, 0.3%). In panel b and d, cells were treated with thapsigargin in the Ca^{2+}-free medium, as detailed in panel a and c, before $CaCl_2$ addition. The results are representative of at least 3 different experiments.

RESULTS

The experimental protocol adopted in the study involved (i) the activation of capacitative Ca^{2+} inflow by emptying the endoplasmic reticular Ca^{2+} pool with thapsigargin [9] in a nominally Ca^{2+}-free medium and (ii) readmission of Ca^{2+} via a bolus of the ion (see eg. [7]). Experiments shown in Figure 1 compare the actions of econazole and oleic acid on capacitative Ca^{2+} inflow into Ehrlich ascites tumour cells.

Fig. 1a shows that bovine serum albumin restores capacitative Ca^{2+} inflow which previously was blocked by econazole. Bovine serum albumin addition did not modify the latter Ca^{2+} inflow in control cells, ie. in the absence of econazole (not shown, but see [4]). In a second experiment (Fig. 1b) econazole was added following Ca^{2+} readmission to block activated capacitative Ca^{2+} inflow; this block was similarly reversed by bovine serum albumin. In Fig. 1c and 1d, econazole was replaced by oleic acid and qualitatively similar effects of bovine serum albumin were observed.

Fig. 2 shows the ability of a fixed concentration of econazole (5μM) to inhibit capacitative Ca^{2+} inflow varies with the concentration of cells. As observed with unsaturated fatty acids [4], the higher the concentration of cells the lower the degree of inhibition induced by econazole.

DISCUSSION

Experiments reported here show that the action of both unsaturated fatty acids and econazole on capacitative Ca^{2+} inflow are qualitatively similar in that they are reversed by bovine serum albumin. In the case of oleic acid we established earlier that bovine serum albumin removed the lipophile bound/inserted into the plasma membrane and that this was sufficient to eliminate its inhibitory action. Econazole is known also to be poorly water-soluble and one would expect its physical behaviour to qualitatively resemble that of fatty acids. The latter are known to insert readily into biological membranes and thereby influence their structure/fluidity [10]. This non-specific effect at the plasma membrane, replicated by other chemically unrelated lipophiles [4], was assumed by us to underlie the inhibitory action of unsaturated fatty acids on capacitative Ca^{2+} inflow [6]. In this context it is of interest that Christian et al [11] observed that econazole did not inhibit I_{CRAC} (assumed to be the ionic current corresponding to ca-

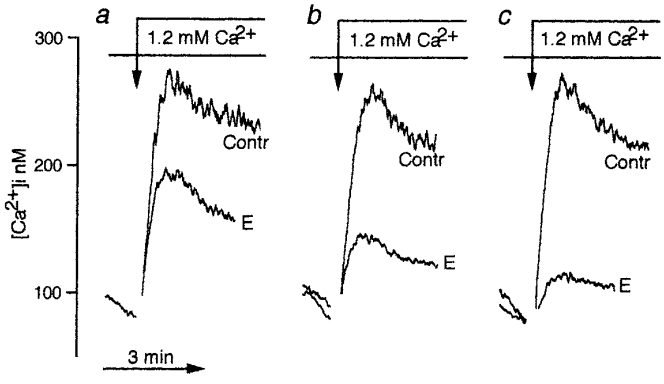

FIG. 2. Inhibition of thapsigargin-induced capacitative Ca^{2+} inflow by econazole in suspensions containing different concentrations of Ehrlich ascites tumour cells. Cells were incubated and treated with thapsigargin as detailed in Fig. 1a. Cell concentrations (10^6 cells/ml) were: 2.5 (a), 1.25 (b), and 0.75 (c). Six min after thapsigargin, $CaCl_2$ (Ca^{2+}, 1.2 mM) was added to the medium. Cells were pre-treated with 5 μM econazole (E) 1 min before Ca^{2+} addition as indicated. The results are representative of at least 3 different experiments.

pacitative Ca^{2+} inflow) when injected intracellularly but did so when presented externally by the patch pipette.

A further similarity between the actions of fatty acids and econazole relates to the dose-dependency. The effect of both compounds on capacitative Ca^{2+} inflow was found to be a function of the ratio of cell mass to inhibitor concentration (see Fig. 2 and [4]). Related to this is the point that bovine serum albumin is known to bind to a variety of exogenous and endogenous lipophilic compounds via interactions that involve relatively low-affinity binding.

With the above considerations in mind, it seems to us unlikely that the inhibitory action of econazole on capacitative Ca^{2+} inflow is one involving a high-affinity specific interaction with the channel components.

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