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Effects of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) on agonist-stimulated Ca^{2+} inflow across the hepatocyte plasma membrane

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Liver cells possess two systems which facilitate the movement of Ca^{2+} across the plasma membrane [1, 2]. These are a basal system, which is responsible for Ca^{2+} inflow in the absence of agonists, and a receptor-activated Ca^{2+} inflow system. Although receptor-activated Ca^{2+} inflow systems play an important role in Ca^{2+} homeostasis in many cell types [3], very little is known about the structure and function of these systems. This contrasts with the large body of knowledge on the structure and function of voltage-operated Ca^{2+} channels present in excitable cells [4]. Previous studies conducted in this laboratory have provided evidence that the receptor-activated Ca^{2+} inflow system in the liver cell is a Ca^{2+} channel, in which the properties of the pore through which Ca^{2+} moves are similar to those of the pores of voltage-operated Ca^{2+} channels [2, 5]. It has been proposed that the latter possess free carboxyl groups which bind Ca^{2+} [4, 6]. Such Ca^{2+}-binding groups are also present in the Ca^{2+}-ATPase of the sarcoplasmic reticulum [7]. Surprisingly, although the role of Ca^{2+}-binding sites in the movement of metal ions through voltage-operated Ca^{2+} channels has been discussed extensively [6], there have been few direct studies of the nature of the Ca^{2+}-binding site. The aim of the present experiments was to test the role of free carboxyl groups in the receptor-activated Ca^{2+} inflow process in hepatocytes. The experimental approach employed has involved a test of the ability of a carboxyl-group-specific agent, *N*-ethyoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ*) [8], to inhibit Ca^{2+} inflow stimulated by vasopressin, angiotensin II or adrenaline.

Materials and Methods

The isolation of hepatocytes from fed male rats, incubation of stirred suspensions of cells in cylindrical chambers and the assessment of cell viability by Trypan Blue exclusion were performed as described previously [1]. Except where indicated otherwise, the incubation medium contained 117 mM NaCl, 4.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 20 mM Tes/KOH, pH 7.4 (Medium A), and hepatocytes [2×10^6 cells (30 mg wet wt)/mL]. The procedures for the treatment of cells with EEDQ were based on those described by others [9, 10]. Hepatocytes were washed twice in 154 mM NaCl, 10 mM Hepes/KOH pH 7.4 and resuspended in this medium (25–30 mg wet wt/mL). Suspensions of cells (9.0 mL volume) were stirred in cylindrical chambers at room temperature and EEDQ, dissolved in dimethylsulphoxide, was added. Control incubations received an equal volume of dimethylsulphoxide. The maximum final concentration of dimethylsulphoxide was 1% (v/v). When present, glycinamide (dissolved in water) was added at the same time as EEDQ. At the desired times the cells were collected by centrifugation at 50 *g* for 1 min, washed once with Medium A in order to remove unreacted EEDQ and resuspended in this medium. Subsequent incubations were performed immediately.

Rates of plasma membrane Ca^{2+} inflow were estimated as described previously [2, 5, 11] by the measurement of (i) the initial rate of activation of glycogen phosphorylase *a* following the addition of extracellular Ca^{2+} (Ca_o^{2+}) to cells previously incubated in the absence of added Ca_o^{2+} and in the presence of an agonist [2], (ii) the inflow of Mn^{2+} across the plasma membrane monitored by measuring the quenching of the fluorescence of intracellular quin2 [5] and (iii) the initial rates of increase in the intracellular concentration of Ca^{2+} bound to quin2 following addition of Ca^{2+} to cells loaded with quin2 and incubated in the absence of added Ca^{2+} [11]. The loading of hepatocytes with quin2 and calculation of free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were performed as described previously [11]. Measurement of the amount of $^{45}Ca^{2+}$ associated with cells incubated in the presence of 0.1 mM $^{45}Ca^{2+}$ was conducted as described previously [1]. Except where indicated otherwise, the results are means ± SEM of the numbers of experiments indicated. Degrees of significance were determined by Students' *t*-test for paired samples. Values of $P > 0.05$ were considered to be not significant.

N - Ethoxycarbonyl - 2 - ethoxy - 1,2 - dihydroquinoline (EEDQ) and glycinamide were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals were of the highest grade available and were obtained from the sources described previously [1, 2, 5, 11].

Results and Discussion

Treatment with EEDQ (1 mM for 30 min) had no effect on the amounts of glycogen phosphorylase activity present in hepatocytes or on the shape of plots of glycogen phosphorylase activity as a function of time obtained following the addition of glucagon (170 nM) or A23187 (10 μM) to cells incubated in the presence of 1.3 mM Ca_o^{2+} (results not shown). These results indicate that treatment of hepatocytes with EEDQ does not inhibit the activity of glycogen phosphorylase or the abilities of increased intracellular concentrations of cyclic AMP or Ca^{2+} to activate glycogen phosphorylase kinase. The viability (assessed by Trypan Blue exclusion) of cells treated for 30 min with either 1 mM EEDQ or 1% (w/v) dimethylsulphoxide was 77 ± 3 and 77 ± 2% (means of four determinations made with two cell preparations), respectively, compared with a value of 90–95% for freshly-isolated hepatocytes. These results indicate that, although treatment of cells with EEDQ led to a decreased viability, this was due to the incubation conditions and not to the action of EEDQ.

Treatment of cells with 1 mM EEDQ for 30 min had no effect on the angiotensin II-induced increase in the initial rate of Ca^{2+}-stimulated glycogen phosphorylase activity (Fig. 1a). EEDQ treatment caused a small inhibition in the initial rate of Ca^{2+}-stimulated glycogen phosphorylase activity in cells subsequently incubated in the absence of an agonist (Fig. 1a). EEDQ treatment also had no effect on the angiotensin II-induced increases in the initial rate of Ca^{2+}-stimulated glycogen phosphorylase activity when a submaximal concentration of angiotensin II (100 nM) was employed (results not shown). The inability of EEDQ to inhibit the angiotensin II-induced increase in the initial rate of Ca^{2+}-stimulated glycogen phosphorylase activity could have been due to the absence, from the site of the reaction, of a suitable nucleophile with which activated carboxyl group intermediates can react [12]. In order to test this possibility cells were treated with EEDQ in the

* Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; Ca_o^{2+}, concentration of extracellular Ca^{2+}; $[Ca^{2+}]_i$, concentration of free cytoplasmic Ca^{2+}.

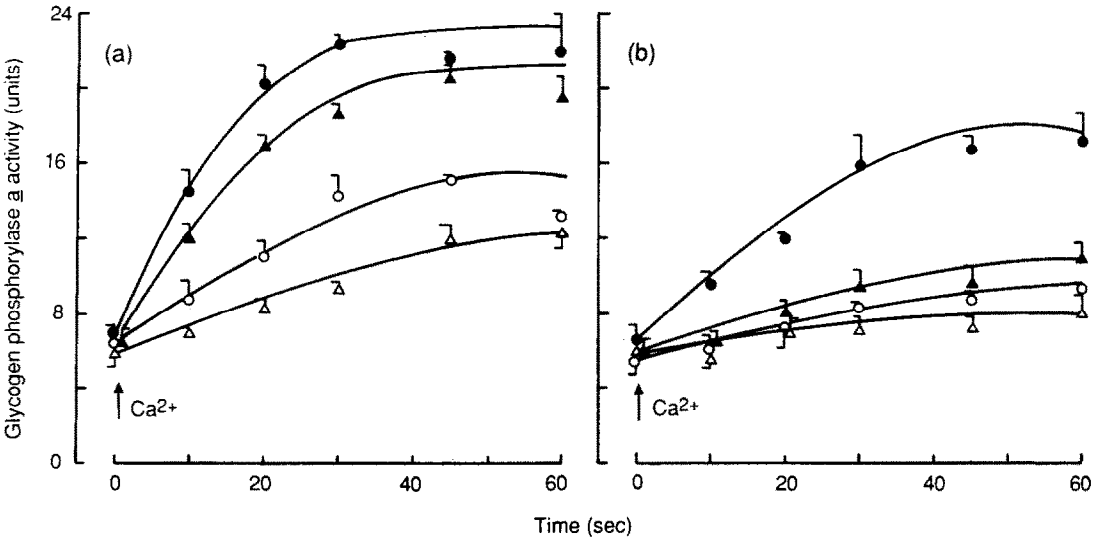

Fig. 1. Effect of pre-treatment of hepatocytes with EEDQ on the Ca^{2+}-induced activation of glycogen phosphorylase. Cells were treated for 30 min with either 1% (v/v) dimethylsulphoxide (○, ●) or 1 mM EEDQ (△, ▲), washed and subsequently incubated in the absence of added Ca^{2+} for 15 min before the addition of either (a) 1 μM angiotensin II or (b) 10 nM vasopressin (▲, ●) or vehicle (○, △), as described in the text. After a further 5 min, 1.3 mM Ca^{2+} was added as indicated by the arrow. Samples were taken for the measurement of glycogen phosphorylase at the indicated times. The data are the means ± SEM of the results of three experiments each conducted with a separate preparation of hepatocytes.

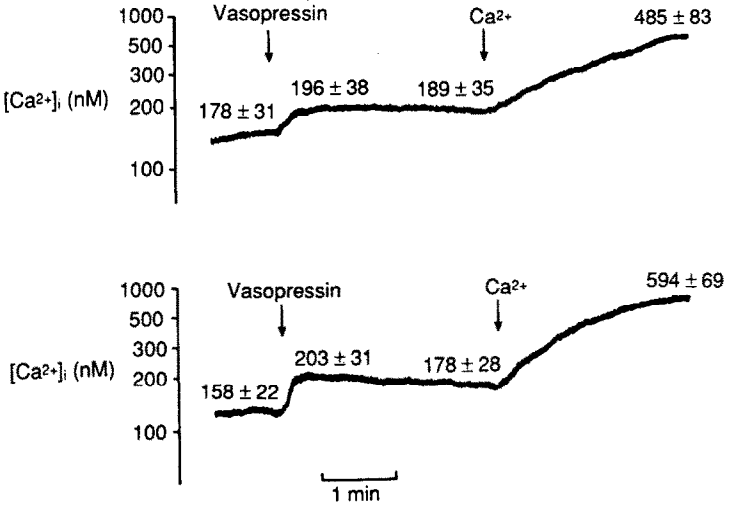

Fig. 2. Effect of pre-treatment with EEDQ on changes in the fluorescence of quin2-loaded cells induced by vasopressin and Ca^{2+}. Cells were treated for 30 min with either 1% (v/v) dimethylsulphoxide (lower trace) or 2 mM EEDQ (upper trace), washed, loaded with quin2 as described in the text, and incubated in the absence of added Ca^{2+}. Vasopressin (10 nM) and $CaCl_2$ (1.3 mM) were added as indicated by the arrows. The numbers on top of each trace are the values of $[Ca^{2+}]_i$ (nM; means ± SEM, N = 3) at that time point, calculated as described in the text. The traces are representative of the results obtained with three preparations of hepatocytes. EEDQ had no substantial effect on the vasopressin-induced increase in autofluorescence in cells not loaded with quin2 (results not shown).

presence of glycinamide [12]. However, treatment of cells with 1 mM EEDQ and 2 mM glycinamide for 30 min had no effect on the angiotensin II-induced increase in the initial rate of Ca^{2+}-stimulated glycogen phosphorylase activity (results not shown).

In contrast to the results obtained with angiotensin II, EEDQ inhibited by 50% the vasopressin-induced increase in the initial rate of Ca^{2+}-stimulated glycogen phosphorylase activity (Fig. 1b) and, in cells incubated in the presence of 1.3 mM Ca_0^{2+}, completely inhibited the ability of adrenaline (10 μM) to stimulate glycogen phosphorylase (results not shown). Further evidence for inhibition of EEDQ of vasopressin-stimulated Ca^{2+} inflow was provided by the observations that treatment of cells for 30 min with a high

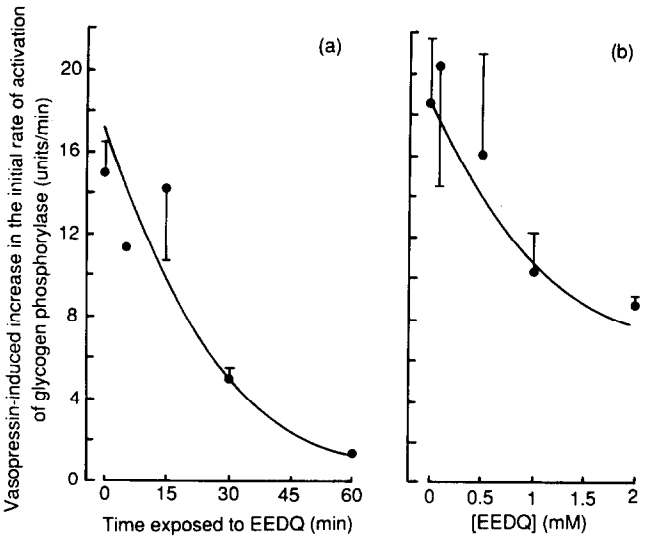

Fig. 3. Time-course (a) and concentration-dependence (b) for the effect of EEDQ on the Ca^{2+}-induced activation of glycogen phosphorylase. (a) Cells were treated with 1% (v/v) dimethylsulphoxide (t = 0 min) or with 1 mM EEDQ for the times indicated as described in the text. The increase in the initial rate of glycogen phosphorylation activation induced by 10 nM vasopressin (●) was determined by subtraction of the rate obtained in the absence of vasopressin from that obtained in the presence of vasopressin. The data are the means ± SEM of the results of 3–10 experiments each conducted with a separate preparation of hepatocytes or the results from one experiment (the 5 or 60 min time points). (b) Cells were treated for 30 min with the indicated concentration of EEDQ in the presence of a final concentration of 1% (v/v) dimethylsulphoxide as described in the text. The increase in the initial rate of glycogen phosphorylase activation induced by 10 nM vasopressin (●) was determined as described in (a). The data are the means ± SEM of the results of 3–6 experiments each conducted with a separate preparation of hepatocytes or the mean ± range of two experiments each conducted with a separate cell preparation (2 mM EEDQ).

concentration of EEDQ, 2 mM, completely inhibited the ability of vasopressin to stimulate the rate of Mn^{2+}-induced quenching of quin2 (results not shown) and reduced the rate of Ca^{2+}-induced increase in quin2 fluorescence measured in the presence of vasopressin (Fig. 2). The rate of Ca^{2+} inflow calculated from these data was reduced from 0.17 ± 0.03 for cells treated with dimethylsulphoxide in place of EEDQ to 0.12 ± 0.02 nmol/min/mg wet wt (mean ± SEM, $N = 3$, $P < 0.05$) for cells treated with EEDQ.

The inhibition by EEDQ of the vasopressin-induced increase in the initial rate of Ca^{2+}-stimulated glycogen phosphorylase activity was slow to develop with maximum inhibition observed at 60 min exposure to EEDQ (Fig. 3a). Half-maximal inhibition was observed at about 0.5 mM EEDQ (Fig. 3b). Concentrations of EEDQ up to 2 mM did not completely inhibit the vasopressin-induced increase in the initial rate of Ca^{2+}-stimulated glycogen phosphorylase activity (Fig. 3b).

The inhibition of vasopressin-stimulated Ca^{2+} inflow caused by treatment of cells with 2 mM EEDQ was associated with a 60% inhibition of the maximum increase in $[Ca^{2+}]_i$ induced by the addition of vasopressin to quin2 loaded cells (Fig. 2). Treatment of cells for 30 min with 1 mM EEDQ caused a 25% inhibition of the maximum increase induced by vasopressin in glycogen phosphorylase activity (results not shown) in cells incubated in the absence of added Ca_0^{2+}, and reduced by 25% the ability of vasopressin to decrease the amount of $^{45}Ca^{2+}$ associated with cells incubated in the presence of 0.1 mM Ca_0^{2+} (results not shown). Treatment with EEDQ did not alter the ability of angiotensin II to activate glycogen phosphorylase in cells incubated in the absence of added Ca_0^{2+} (results not shown) and completely inhibited the activation caused by adrenaline (results not shown). These results indicate that EEDQ inhibits the ability of adrenaline and vasopressin to release Ca^{2+} from internal stores. Taken together with the observations that in other cell types EEDQ inhibits the binding of catecholamines to their receptors [8, 13], the present results suggest that the most likely explanation for the inhibition by EEDQ of adrenaline- and vasopressin-stimulated Ca^{2+} inflow is the interaction of EEDQ with the extracellular domains of the adrenaline and vasopressin receptors.

On the basis of the observation that angiotensin II-stimulated Ca^{2+} inflow is not inhibited by EEDQ and the evidence reported previously which indicates that angiotensin II, vasopressin and adrenaline each activate the same receptor-operated Ca^{2+} channel [2, 14], it is concluded that EEDQ does not inhibit the movement of Ca^{2+} through the receptor-operated Ca^{2+} channel in the liver cell plasma membrane. It is considered that the most likely explanation for the data obtained with angiotensin II is that carboxyl groups which play a necessary role in the inward movement of Ca^{2+} are present in the channel but are not accessible to EEDQ. An alternative explanation, that the channel does not possess such carboxyl groups, cannot be excluded, although it is considered to be unlikely. The present conclusion that carboxyl groups in the liver cell receptor-operated calcium channel activated by angiotensin II are not accessible to EEDQ differs from the observations of Karjalainen and Bygrave [15] that EEDQ inhibits the ability of the combination of glucagon and vasopressin to stimulate Ca^{2+} inflow in hepatocytes by an inhibition of the Ca^{2+} inflow channel. The apparent disagreement between the conclusions may be due to the

nature of the agonists used to stimulate Ca^{2+} inflow and to substantial differences in the incubation conditions employed for the treatment of cells with EEDQ.

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Purification of methylglyoxal bis(guanylhydrazone)-induced spermidine *N*-acetyltransferase from baby hamster kidney cells (BHK-21/C13)

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Acetylation is the rate-limiting step in the degradation of spermine to spermidine and spermidine to putrescine [1]. These naturally occurring polyamines are essential for cell growth, and it is thought that acetylation is a means whereby the cell can decrease its intracellular polyamine content under conditions of growth inhibition [2, 3]. Distinct acetyltransferase enzymes, characterized by other substrate specificities, are present in the cell cytosol and nucleus [4, 5]. Two nuclear *N*-acetyltransferase enzymes have been purified from calf liver. These enzymes acetylated both spermidine and spermine [6]. A carbon tetrachloride-induced cytosolic acetyltransferase enzyme has also been purified from rat liver. This enzyme is distinct from the nuclear one and also acetylates spermidine and spermine [7].

Methylglyoxal bis(guanylhydrazone) (MGBG), an inhibitor of polyamine biosynthesis, has been shown to stimulate spermidine and spermine acetyltransferase activity in rat liver and kidney [4] and in baby hamster kidney (BHK-21/C13) cells [8]. The drug was used as an anti-leukaemic agent in the 1960s, but its use was discontinued due to toxic side-effects [9]. Recently, however, MGBG has been re-