The early stimulation of glycolysis by epidermal growth factor in isolated rat hepatocytes is secondary to the glycogenolytic effect

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We have studied the relationship between the effect of epidermal growth factor (EGF) on glycogen metabolism and its effect on glycolysis, in rat hepatocyte suspensions. Although 10 nM glucagon or 10 μM adrenaline increased glycogen degradation by more than 120%, 10 nM EGF increased glycogenolysis by less than 20% in hepatocytes incubated in glucose-free medium. Both glucagon and adrenaline increased phosphorylase *a* activity by more than 130%; EGF increased this activity by about 30%. Under basal conditions, 65% of the glucosyl residues were released as free glucose and about 30% ended up as C_3 molecules (lactate and pyruvate). Both glucagon and adrenaline decreased the proportion of glucosyl units that rendered glycolysis end-products (to 2% for glucagon and 6% for adrenaline) and increased the proportion that ended up as free glucose (to 94% and 88% of the glucosyl residues for glucagon and adrenaline respectively). EGF increased the production of both free glucose and lactate + pyruvate, but the proportion of glucosyl residues that ended up as free glucose or glycolysis end-products was unchanged. In glycogen-depleted hepatocytes incubated in the presence of 25 mM glucose, EGF affected neither glycogen deposition nor glycolysis. EGF increased cytosolic free Ca^{2+}, and neomycin decreased both the Ca^{2+} signal and the glycogenolytic effect. In conclusion, our results indicate that the effect of EGF on glycolysis is secondary to the Ca^{2+}-mediated stimulation of glycogenolysis in rat hepatocyte suspensions.

INTRODUCTION

In addition to the effect of epidermal growth factor (EGF) on cell proliferation [1], several studies demonstrate that it regulates various metabolic events in different cell types. In adipocytes, EGF stimulates acetyl-CoA carboxylase and increases lipogenesis [2], and interferes with the lipolytic action of catecholamines and glucagon [3]. In both perfused livers [4] and isolated hepatocytes [5] from fasted rats, EGF increased gluconeogenesis in a rapid but transient manner. Some delayed effects on gluconeogenesis were also reported [6–8], but they were secondary to the effect of EGF on cell redox state [5].

In hepatocytes from fed rats, it was reported that EGF, like insulin, stimulates glycogen synthesis [9]; other reports indicate that EGF counteracts the glycogenic effect of insulin [10–12]. Furthermore, it was recently reported that EGF, by itself, inhibited glycogen deposition in cultured hepatocytes [12]. Concerning enzyme activities, it was reported that EGF increases both glycogen synthase [9] and glycogen phosphorylase *a* [13] activities.

In addition to the effect on glycogen metabolism, it has been described that EGF stimulates glycolysis and the pentose phosphate pathway in isolated hepatocytes [14]. Since the effect of EGF was apparent even in glucose-free medium, we studied the relationship between the effect of EGF on glycogen metabolism and its effect on glycolysis. Our results indicate that the action on glycolysis is the consequence of the stimulation of glycogen breakdown.

EXPERIMENTAL

Male Wistar rats (200–250 g) were used. Hepatocytes from fed animals were isolated by the collagenase-perfusion method of Berry and Friend [15] as previously described [5]. Initial cell viability measured by the Trypan Blue exclusion test was over 90%, and decreased by about 10% during incubation (60 min). Hormones did not affect this decrease.

Isolated hepatocytes [$(1–3) \times 10^6$ cells/ml; final volume 2 ml] were incubated in a 20 mM Hepes (pH 7.4)-containing buffer supplemented with 1% BSA [11], but without glucose (buffer A). Incubation was carried out with constant shaking (80 cycles/min) at 37 °C under O_2/CO_2 (19:1). In these conditions the pH was maintained at 7.4 during the whole incubation period. For metabolite assays, at the end of the incubation, a sample of the suspension was placed into enough ice-cold $HClO_4$ to give a final concentration of 6%. After neutralization, glucose [16], glycogen [17], lactate [18] and pyruvate [19] concentrations were determined. Cyclic AMP was determined in $HClO_4$ extracts as in [3]. In preliminary experiments the cells were separated from the medium by centrifugation before addition of $HClO_4$. Since there were no differences between this and the procedure described above, we deproteinized the whole sample in all experiments described here.

Cytosolic free Ca^{2+} was measured in Fura-2/AM-loaded hepatocytes. Isolated hepatocytes (3×10^6 cells/ml) were incubated in buffer A supplemented with 5.5 mM glucose, amino acids, and vitamins (composition given as buffer D in [20]) for 45 min at 37 °C in the presence of 5 μM Fura-2/AM. The cells were then rinsed three times with fresh Fura-2/AM-free medium and further incubated for 15 min to allow de-esterification of the dye. Cells were rinsed twice with fresh medium before their fluorescence was monitored in a Shimadzu RF5001PC spectrofluorimeter (excitation at 340 and 380 nm and emission at 505 nm). At the end of each experiment, 0.06 ml of Triton X-100 and 0.1 ml of 100 mM EGTA were sequentially added. Cytosolic Ca^{2+} was quantified as in [21].

To determine enzyme activities, a sample was taken at the indicated times and centrifuged (30 s at 10000 *g*, 4 °C). The medium was discarded and the cells were immediately frozen in liquid N_2. Cell pellets were sonicated in ice-cold extraction buffer

Abbreviation used: EGF, epidermal growth factor.
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[for pyruvate kinase: 20 mM Tris/HCl (pH 7.5)/120 mM KCl/5 mM $MgSO_4$/0.1 mM EDTA/10 mM NaF; for glycogen phosphorylase *a*: 40 mM glycerol 2-phosphate (pH 6.8)/40 mM β-mercaptoethanol/10 mM NaF/0.1% albumin]. After centrifugation at 10000 *g* for 5 min, the supernatant was immediately used for assays. The pyruvate kinase activity ratio (without/with 0.12 mM fructose 1,6-bisphosphate) was determined at 25 °C as in [22] at 0.6 mM phosphoenolpyruvate. Glycogen phosphorylase *a* activity was determined in the direction of glucose 1-phosphate release from glycogen at 30 °C [23]. One unit of enzyme activity was defined as the amount of enzyme that catalysed the release of 1 μmol of glucose 1-phosphate. Cell proteins were determined as in [24].

Fura-2/AM was obtained from Boehringer-Mannheim (Mannheim, Germany). All other chemicals used were as described elsewhere [3,5,11,25].

RESULTS AND DISCUSSION

In a preliminary experiment we studied the effect of EGF on glycolysis (lactate + pyruvate production) in hepatocytes incubated in the presence of increasing concentrations of glucose. Glycolysis increased with the glucose concentration in the medium (86 ± 7, 276 ± 19 and 394 ± 21 nmol of lactate + pyruvate/10^6 cells at 0, 15 and 25 mM glucose in medium respectively). EGF increased lactate + pyruvate production at any glucose concentration studied (138 ± 8, 357 ± 15 and 437 ± 27 nmol of lactate + pyruvate/10^6 cells at 0, 15 and 25 mM glucose in medium respectively). The relative effect of EGF decreased as the glycolytic flux increased (1.60-, 1.29- and 1.11-fold increase respectively). Similar results were obtained by Conricode and Ochs [14].

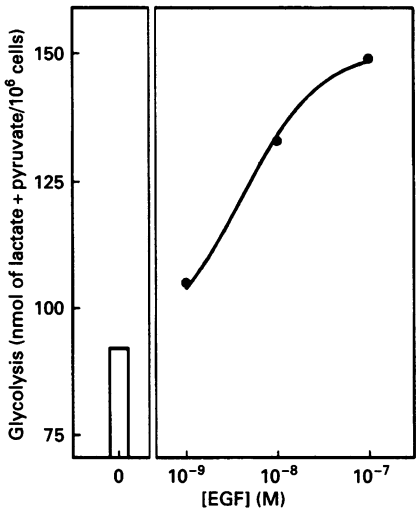

Figure 1 Dose-dependent effect of EGF on glycolysis

Hepatocytes were incubated for 30 min in a glucose-free medium and in the presence of increasing concentrations of EGF. At the end of the incubation a sample was taken to determine lactate + pyruvate. Results are means of duplicate values from a representative experiment.

The relationship between the EGF concentration and glycolysis was studied in glucose-free medium, because under this condition the relative effect of EGF was the highest. As shown in Figure 1, EGF increased glycolysis in a dose-dependent manner. The ED_{50} obtained (4.0 nM) was in keeping with the values reported for several effects of EGF in hepatocytes [9,26–28].

Since in glucose-free medium the only source of glucosyl units for glycolysis is glycogen, we studied the relationship between glycogen degradation, glucose output and glycolysis in this condition. As shown in Table 1, about 65% of the glucosyl residues released from glycogen were recovered as free glucose and only 29% were recovered as lactate + pyruvate. The glycogen unaccounted for (which always represents less than 3% at 30 min and less than 12% at 60 min of the glycogen degraded) may represent oxidation either through the pentose phosphate pathway or through mitochondrial oxidation, which includes decarboxylation in the pyruvate dehydrogenase reaction and in the tricarboxylic acid cycle. Similar results were obtained in preliminary experiments where 5 mM glucose was added to the medium. Since the glucose release was easier to determine in the absence of added glucose, the following experiments, unless otherwise indicated, were performed in glucose-free medium.

Glucagon increased glycogen mobilization by 120%, and decreased lactate + pyruvate production (Table 1). Consequently, glucagon increased the proportion of glucosyl units released as free glucose to near 95%. Adrenaline produced similar effects; it stimulated glycogen mobilization, increased glucose release, and inhibited glycolysis. Since the effect of adrenaline on glycolytic flux is less potent than that of glucagon, the proportion of glucosyl units released as free glucose was somewhat lower (88%).

EGF produced a moderate but consistent glycogenolytic effect (Table 1). Both glucose release and glycolysis were increased in hepatocytes incubated with EGF; as a result, the proportion of

Table 1 Effect of EGF on glycolysis from endogenous sources

Isolated hepatocytes were incubated in glucose-free medium in the presence of EGF (10 nM), glucagon (10 nM) or adrenaline (10 μM). At 30 and 60 min of incubation a sample was taken for assays. Results are means ± S.E.M. of 12 experiments with different cell preparations. Statistical comparisons versus basal were made by paired Student's *t* test: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Initial glycogen content of isolated hepatocytes was 2.08 ± 0.1 μmol of glucose residues/10^6 cells ($n = 12$).

	Glycogen mobilization (nmol of glucose residues/10^6 cells)		Glucose release (nmol of glucose/10^6 cells)		Glycolytic flux [nmol of (lactate + pyruvate)/10^6 cells]	
Time (min)...	30	60	30	60	30	60
Control	439 ± 71	769 ± 126	299 ± 42	467 ± 62	261 ± 18	421 ± 49
EGF	502 ± 82*	885 ± 140*	344 ± 44***	525 ± 65*	313 ± 23***	493 ± 58**
Glucagon	1019 ± 126***	1615 ± 290**	972 ± 99***	1500 ± 208***	54 ± 12***	49 ± 25***
Adrenaline	1001 ± 145***	1672 ± 310**	899 ± 119***	1442 ± 214***	156 ± 15***	145 ± 18***

Table 2 Effect of low concentrations of glucagon on glycogen degradation and glucose release

Isolated hepatocytes were incubated in glucose-free medium in the presence of EGF or increasing concentrations of glucagon. At 30 min of incubation a sample was taken for assays. Results are mean ± S.E.M. of four experiments with different cell preparations. Statistical comparisons versus basal were made by paired Student's *t* test: ns, non-significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

	Glycogen degradation (nmol of glucose residues/10^6 cells)	Glucose release (nmol of glucose residues/10^6 cells)
Control	674 ± 48	419 ± 26
EGF (10 nM)	723 ± 50**	480 ± 30**
Glucagon		
(0.01 nM)	675 ± 40 ns	449 ± 27*
(0.1 nM)	657 ± 44 ns	479 ± 39**
(1 nM)	882 ± 59***	843 ± 31***
(10 nM)	1184 ± 85***	1052 ± 25***

Table 3 Effects of EGF on pyruvate kinase and glycogen phosphorylase *a* activities, and lack of effect of EGF on cyclic AMP

Hepatocytes were incubated for 30 min (pyruvate kinase), 3 min (phosphorylase *a*) or 10 min (cyclic AMP) in glucose-free medium and in the absence or in the presence of 10 nM EGF, 10 nM glucagon or 10 μM adrenaline. Pyruvate kinase activity was determined at a phosphoenolpyruvate concentration of 0.6 mM in the assay system, in the absence and in the presence of 0.12 mM fructose-1,6-bisphosphate. Results are means ± S.E.M. of three experiments with different cell preparations. Statistical comparisons versus control value were made by paired Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

	Pyruvate kinase activity (−/+ fructose-1,6-bisphosphate)	Phosphorylase *a* activity (m-units/mg of protein)	Cyclic AMP (pmol/10^6 cells)
Control	0.55 ± 0.02	58 ± 5	4.5 ± 0.4
EGF	0.53 ± 0.02 ns	74 ± 5*	4.6 ± 0.5 ns
Glucagon	0.31 ± 0.01**	170 ± 12***	39.2 ± 3.6***
Adrenaline	0.48 ± 0.02*	141 ± 8**	6.0 ± 0.3*

glucosyl residues released as free glucose (64%) was similar to that in control cells, as was the proportion that entered glycolysis (30%). The effect of EGF on glycogenolysis is similar at 30 and 60 min of incubation, denoting that this effect is not transient, in contrast with what was reported in hepatocytes and perfused livers from fasted rats, where EGF produces a rapid but transient stimulation of gluconeogenesis [5,29]. Our results are in keeping with the recent observation that EGF decreases the deposition of glycogen, and increases lactate production in cultured hepatocytes [12].

In contrast with glucagon or adrenaline, EGF did not affect the proportion of glucosyl residues released from glycogen that entered glycolysis. To determine whether this difference is dictated by the actual degree of glycogenolysis taking place, which is clearly different, we analysed the relationship between glycogen degradation and glucose release at increasing concentrations of glucagon. As shown in Table 2, glucagon increased glucose release at concentrations as low as 0.01 nM, whereas it required concentrations of 1 nM or higher to increase the rate of glycogen degradation. Therefore, the effect of glucagon increasing the proportion of glucosyl residues released as free glucose is the consequence of specific effect(s) on the glycolytic pathway. Studying the phosphorylation of several cytosolic proteins, Connelly et al. [30] found that pyruvate kinase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase were the most sensitive among these proteins to low concentrations of glucagon. Therefore, the effect of very low concentrations of glucagon, increasing glucose release without any increase in glycogen degradation (Table 2), may be the consequence of the inhibition of glycolysis.

The effect of nearly saturating concentrations of glucagon and adrenaline on glycolysis correlates well with the effect of both hormones on pyruvate kinase activity. As shown in Table 3, glucagon inhibited pyruvate kinase activity. Adrenaline also decreased pyruvate kinase activity, but the effect was considerably smaller than that of glucagon. These results are in keeping with those shown in Table 1 concerning glycolysis.

EGF did not affect pyruvate kinase activity (Table 3). We have reported that EGF does not affect lactate production from dihydroxyacetone in hepatocytes from fasted rats [5]. In these cells, where the glycogen content is very low, the only regulatory step in the conversion of dihydroxyacetone into lactate is the pyruvate kinase reaction. Thus, in this condition, EGF did not affect the flux through pyruvate kinase. Nevertheless, other authors have reported effects of EGF on pyruvate kinase activity. Moule and McGivan [27,31] reported a phosphorylation and inhibition of pyruvate kinase in hepatocytes incubated with EGF. Conricode and Ochs [14], however, reported a modest increase in its activity. Rashed and Patel [29] reported a rapid but transient decrease in pyruvate kinase activity in fasted-rat liver perfused with EGF. In our preparations, we could not observe any transient effect of EGF on pyruvate kinase activity (results not shown). Since pyruvate kinase phosphorylation is influenced by allosteric effectors, the different metabolic status of the cells might explain some of these discrepancies.

To determine whether the effect of EGF on glycolysis is a consequence of the effect on glycogen degradation, we studied the effect of EGF on the glycolytic flux in hepatocytes depleted of glycogen. Two models were used. In the first, hepatocytes obtained from fed rats were maintained in a glucose-free medium for 60 min. After this incubation, hepatocytes had lost nearly 50% of the initial glycogen content. The cells were then rinsed with fresh medium and further incubated in the absence or in the presence of 25 mM glucose and with or without 10 nM EGF. EGF was still able to increase glycogen degradation if hepatocytes were incubated in the absence of glucose (Table 4). Glucose output and glycolysis were also higher in the presence of EGF. Hepatocytes partially depleted of glycogen, when incubated in the presence of 25 mM glucose, accumulated instead of degraded glycogen. Under these conditions, EGF had no significant effect either on glycogen deposition or on glucose and lactate + pyruvate production. Similar experiments were performed with hepatocytes isolated from fasted rats. In this second model, hepatocytes had lost more than 85% of the glycogen stored in the fed state. These cells were incubated only in the presence of 25 mM glucose in the medium. Again, these cells accumulated glycogen, and EGF did not affect either glycogen deposition or glucose and lactate + pyruvate production. Therefore, our results strongly suggest that the early effect of EGF on glycolysis is secondary to the effect on glycogenolysis.

Hughes et al. [13] showed that EGF increased glycogen phosphorylase activity. In Table 3 we also show that EGF increased phosphorylase *a* activity, but to a much lesser extent than did adrenaline or glucagon. This is in accordance with the smaller glycogenolytic effect shown in Table 1.

Glycogen phosphorylase is activated by phosphorylase kinase, which is sensitive to both cyclic AMP and Ca^{2+} signals. Since

Table 4 Effect of EGF on glycolysis in glycogen-depleted hepatocytes

Isolated hepatocytes from fed rats were incubated for 60 min at 37 °C in glucose-free medium to induce glycogen depletion. The cells were then rinsed twice with fresh medium and incubated for 30 min in glucose-free or 25 mM-glucose-containing medium in the absence (control) or in the presence of 10 nM EGF. Isolated hepatocytes from 24 h-fasted rats were incubated in 25 mM-glucose-containing medium in the absence or in the presence of EGF. Results are means ± S.E.M. of four experiments with different cell preparations (nd, not determined). Statistical comparisons versus control values were made by paired Student's *t* test (ns, not significant). The glycogen content was 1.61 ± 0.40 and 0.33 ± 0.05 μmol of glucose residues/10^6 cells for glycogen-depleted hepatocytes from fed rats and isolated hepatocytes from fasted rats respectively.

Hepatocytes	Incubation	Δ Glycogen (nmol of glucose residues/10^6 cells)	Glucose release (nmol of glucose/10^6 cells)	Glycolytic flux [nmol of (lactate + pyruvate)/10^6 cells]
Glycogen-depleted	In glucose-free medium			
	Control	−463 ± 22	332 ± 23	240 ± 12
	EGF	−531 ± 46	369 ± 33	295 ± 20
	P vs control	< 0.05	< 0.05	< 0.05
	In 25 mM glucose medium			
	Control	+401 ± 119	nd	400 ± 60
	EGF	+458 ± 165	nd	409 ± 59
	P vs control	ns		ns
From fasted rat	In 25 mM glucose medium			
	Control	+20 ± 4	nd	291 ± 30
	EGF	+17 ± 8	nd	306 ± 35
	P vs control	ns		ns

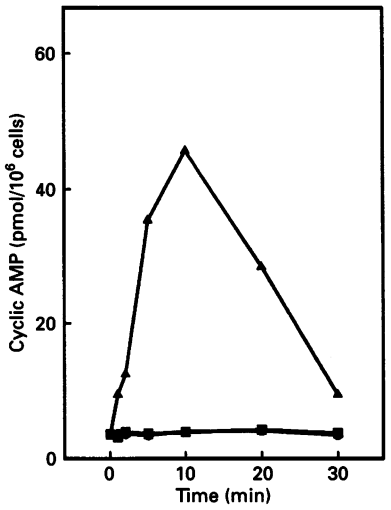

Figure 2 Time course of the effect of glucagon and EGF and cyclic AMP

Hepatocytes were incubated for up to 30 min in glucose-free medium: ●, control; ■, 10 nM EGF; ▲, 10 nM glucagon. Results are means of duplicate values from one experiment.

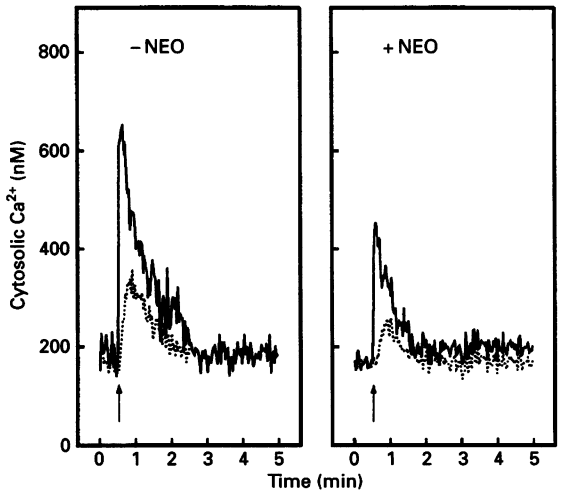

Figure 3 Effect of EGF on cytosolic free Ca^{2+}

Hepatocytes were loaded with Fura-2/AM as indicated in the Experimental section, to determine the cytosolic free Ca^{2+} concentration. At the indicated time (arrow) the cells received adrenaline (final concn. 10 μM) (continuous line) or EGF (final concn. 10 nM) (dotted line). The Figure shows a representative experiment with hepatocytes incubated either in the absence of neomycin (−NEO) or in the presence of 2 mM neomycin since 10 min before hormone additions (+NEO).

there are few reports on the effect of EGF on cyclic AMP in hepatocytes [9,10], and since EGF has different effects on this messenger, depending on the cell type [32–36], we measured the effect of EGF on cyclic AMP levels in our hepatocyte preparations. As shown in Figure 2, glucagon rapidly increased cyclic AMP levels in hepatocytes. The peak (775% above control value) was achieved 10 min after hormone addition. Adrenaline also increased cyclic AMP levels (Table 3), but to a much lesser extent (33% above control value). Note that we isolated hepatocytes from the liver of adult male rats, where the cyclic AMP response to catecholamines is much lower than in hepatocytes from juvenile or female rats [37]. The increase in cyclic AMP appears to be linked to both α_1- and β_2-adrenergic receptors in adult male hepatocytes [38].

EGF did not increase cyclic AMP at any time studied (Figure 2 and Table 3). This is in agreement with other reports [9,10]. Therefore, the glycogenolytic effect of EGF does not appear to be mediated by any increase in cyclic AMP. Many reports agree that EGF causes an increase in cytosolic Ca^{2+} levels in hepatocytes [9,13,39–44], and we have observed also that EGF increases cytosolic free Ca^{2+} concentration in fura-2-loaded hepatocytes, but to a lower level than with adrenaline (Figure 3). Johnson et

Table 5 Effect of neomycin on EGF-stimulated glycogenolysis

Isolated hepatocytes were incubated in the absence or in the presence of 2 mM neomycin (NEO). After 10 min hormones were added to give final concentrations of 10 nM EGF, 50 nM adrenaline or 0.5 nM vasopressin. At this time and 30 min afterwards samples were taken to determine glucose production. Results are means ± S.E.M. of four experiments with different cell preparations. Statistical comparisons between neomycin and control values were made by paired Student's *t* test: $^{**}P < 0.01$.

	Glycogen degradation (nmol of glucose residues/10^6 cells)	
	−NEO	+NEO
Control	212 ± 5	196 ± 8
EGF	261 ± 5	222 ± 7**
Adrenaline	490 ± 4	453 ± 4**
Vasopressin	360 ± 4	295 ± 9**

al. [41] found that not only was the peak Ca^{2+} concentration smaller for EGF than for angiotensin-II, but also the time to reach the peak was longer for EGF. We obtained a time to the Ca^{2+} peak of 3.0 ± 0.2 s when hepatocytes were exposed to adrenaline, and 15.0 ± 0.8 s when they were exposed to EGF. The effect of EGF on cytosolic Ca^{2+} concentration may be responsible for the stimulation of phosphorylase *a* activity, and thus the increase in glycogen degradation shown here (Table 1). The small extent of the glycogenolytic effect of EGF appears to be in agreement with the moderate effect of EGF on cytosolic Ca^{2+} levels, as compared with the effect of other well-characterized Ca^{2+}-mobilizing hormones [41,42], and with the lack of effect on cyclic AMP.

The Ca^{2+} signal generated by EGF can be decreased by incubating the cells with the phospholipase C inhibitor neomycin [9]. Therefore, to determine whether the glycogenolytic effect of EGF involves the rise in cytosolic Ca^{2+}, we incubated hepatocytes with 2 mM neomycin for 10 min before addition of 10 nM EGF. For comparison, the effect of neomycin on the stimulation of glycogenolysis by 50 nM adrenaline or 0.5 nM vasopressin was also studied. A sub-maximal concentration of hormones was used, because at higher doses the effect of neomycin was decreased (results not shown). Neomycin decreased the rise in cytosolic Ca^{2+} induced by adrenaline or EGF (Figure 3), and also significantly decreased the glycogenolytic effect of EGF, adrenaline and vasopressin (Table 5). However, neither the Ca^{2+} signal nor the glycogenolytic effect of EGF was completely abolished by neomycin. This is in keeping with reports indicating that EGF stimulates both the inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from intracellular stores, and the plasma-membrane Ca^{2+} inflow in adult rat hepatocytes [42,45].

In cultured hepatocytes from fetal rats, EGF increases both [^{14}C]glucose incorporation into glycogen and total cellular glycogen content [46]. This is also an early effect of EGF, since maximal stimulation occurs at 1 h of incubation [46]. The difference between fetal and adult hepatocytes in the effect of EGF on glycogen metabolism may be understood in light of the report by Leoni et al. [47] showing that EGF neither increases inositol trisphosphate production, nor induces a Ca^{2+} wave in fetal hepatocytes.

Besides the early glycogenolytic effect and the interference with glycogen deposition [12], EGF produces a late induction of glucose-6-phosphate dehydrogenase and the suppression of malic enzyme and lipogenesis in adult hepatocytes [48]. All these effects may facilitate the shift of glucose molecules into pentose phosphate pathway to satisfy the needs for deoxyribose for DNA synthesis, which takes place several hours after the exposure to EGF.

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