Anti-diabetic biguanides inhibit hormone-induced intracellular Ca^{2+} concentration oscillations in rat hepatocytes

Joachim J. UBL, Shuhua CHEN and Jörg W. STUCKI*

Pharmakologisches Institut der Universität Bern, Friedbühlstrasse 49, CH-3010 Bern, Switzerland

Rat hepatocytes respond to glycogenolytic stimuli acting via phosphoinositide breakdown (e.g. α_1 -adrenergic agonists, vasopressin) by oscillations of the free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). We have investigated the action of metformin and phenformin, two anti-diabetic drugs of the biguanide type, on phenylephrine-induced $[\text{Ca}^{2+}]_i$ oscillations. Metformin and phenformin lowered the frequency of the $[\text{Ca}^{2+}]_i$ oscillations in a concentration-dependent manner with an IC_{50} of 0.1 mM and 1 μM , respectively. Simultaneous addition of the biguanides and insulin resulted in a further reduction of the frequency. By contrast, agents which increase the cellular cyclic AMP (cAMP) concentration (glucagon, forskolin, $N,2'$ - O -dibutyl-AMP) reversed this inhibition. Furthermore, we investigated whether biguanides influenced the agonist-induced Ca^{2+} influx across the plasma membrane. When hepatocytes were loaded with the acetoxymethyl ester of fura-2 (fura-2/AM), addition of Mn^{2+} led to a quench of cellular fura-2, measured at the isosbestic excitation wavelength of 360 nm, until a new steady state was reached. Surprisingly, however, this addition of Mn^{2+} caused a marked increase of the fluorescence ratio simultaneously measured at 340 and 380 nm during the approach of the 360 nm signal to a new steady state. This observation can be understood on the basis of a compartmentalization of fura-2/AM into

intracellular stores sensing the $[\text{Ca}^{2+}]$ therein. Subsequent application of phenylephrine resulted in a further decline of the fura-2 signal at 360 nm and a concomitant decrease of the fluorescence ratio. This second phase of the Mn^{2+} quench and the decrease of the fluorescence ratio could be diminished by addition of either 3 mM metformin or 30 μM phenformin. By contrast, when hepatocytes were loaded with fura-2/potassium salt via a patch pipette, only the initial Mn^{2+} -induced quench, measured at 360 nm, but no change of the fluorescence ratio, could be observed. The subsequent addition of phenylephrine and biguanides during the on-going quench caused no further changes, except for a fading oscillatory response. After loading hepatocytes with fluo-3 acetoxymethyl ester, the cells were permeabilized with 5 μM digitonin. Addition of inositol-1,4,5-trisphosphate (IP_3) caused a rapid decrease of the remaining cellular fluorescence which could be effectively inhibited by 20 $\mu\text{g}/\text{ml}$ heparin, indicating a release of Ca^{2+} from intracellular compartments mediated by IP_3 . This IP_3 -induced release of Ca^{2+} from intracellular stores could be diminished by prior addition of metformin and phenformin. Therefore we concluded that the biguanides exerted their inhibitory effect on phenylephrine-induced $[\text{Ca}^{2+}]_i$ oscillations by a direct negative interference with the IP_3 -sensitive channel of intracellular Ca^{2+} stores.

INTRODUCTION

For more than 30 years the biguanides metformin (dimethylbiguanide) and phenformin (phenethylbiguanide) have been used as glucose-lowering agents in the treatment of non-insulin-dependent diabetes mellitus (NIDDM). During the 1970s phenformin was withdrawn in many countries because of an association with lactic acidosis. In spite of intensive research the mechanism of the anti-hyperglycaemic action of biguanides is still a matter of debate (for review see ref. 1).

In contrast to sulphonylureas, the glucose-lowering effect of biguanides occurs without stimulation of insulin secretion [2] and results mainly from improved basal and insulin-mediated glucose utilization in peripheral tissues such as muscle and adipose tissue [3,4]. In liver cells high concentrations of metformin ($> 10^{-3}$ M) reduce basal and glucagon-stimulated gluconeogenesis in the absence of added insulin [5,6]. However, therapeutic concentrations of metformin (10^{-5} M) act synergistically with physiological concentrations of insulin to reduce gluconeogenesis in isolated rat hepatocytes [7,8], indicating a requirement of insulin for its

therapeutic effect. Despite the fact that in a number of studies an enhancement of insulin binding to its receptor on target cells by metformin is reported [9–13], only a poor correlation exists between the effects of metformin on glucose metabolism and the increased insulin binding [14]. This suggests that the potentiation of the insulin action by biguanides occurs distal to insulin receptor binding. In rat hepatocytes metformin did not alter the binding of insulin, but decreased the stimulatory action of glucagon and $N,2'$ - O -dibutyl-cyclic AMP (db-cAMP) on gluconeogenesis, indicating an effect even at a post-adenylate cyclase step [15]. Moreover metformin increased the insulin-dependent stimulation of glycogen and lipid synthesis directly without modification of the cell sensitivity to the hormone [16].

In conclusion, these results indicate that at least part of the anti-hyperglycaemic action of biguanides arises from an alteration of insulin-mediated carbohydrate metabolism (e.g. gluconeogenesis, glycogen synthesis) at a post-receptor level.

It is well established that for a wide variety of cell types the activation of receptors coupled to the breakdown of phosphoinositide (PI) results in oscillations of the free intracellular Ca^{2+}

Abbreviations used: $[\text{Ca}^{2+}]_i$, free intracellular Ca^{2+} concentration; cAMP, cyclic AMP; db-cAMP, $N,2'$ - O -dibutyl-AMP; FCS, foetal calf serum; fura-2/AM, fura-2 acetoxymethyl ester; fluo-3/AM, fluo-3 acetoxymethyl ester; NIDDM, non-insulin-dependent diabetes mellitus; PI, phosphoinositide; $[\text{cAMP}]_{\text{cyt}}$, cytosolic cAMP concentration; IP_3 , inositol 1,4,5-trisphosphate; PKA, cAMP-dependent protein kinase.

* To whom correspondence should be addressed.

concentration ($[Ca^{2+}]_i$) [17]. In rat hepatocytes $[Ca^{2+}]_i$ oscillations occur in response to glycogenolytic agonists (α_1 -adrenergic agonists, vasopressin) [18,19]. Besides the elevation of $[Ca^{2+}]_i$ the cell possesses many other routes for intracellular signalling and it may be assumed that the cellular response to extracellular stimuli is the result of a complex cross-talk among the different signalling pathways. In hepatocytes the Ca^{2+} elevating response induced by agonists acting via PI breakdown has been augmented by agents increasing the cytoplasmic concentration of cAMP [glucagon, forskolin, cyclic AMP (cAMP) analogues] [20–22]. The elevation of [cAMP] by different agonist alone did not induce $[Ca^{2+}]_i$ oscillations in rat hepatocytes but exerted a positive modulatory effect on phenylephrine-induced $[Ca^{2+}]_i$ spikes [23]. This synergism reflects the common metabolic goal of these agonists [20]. In addition insulin, the physiological counterpart to the gluconeogenic agonists, has been shown to suppress phenylephrine-induced $[Ca^{2+}]_i$ oscillations [23].

A prerequisite for sustained $[Ca^{2+}]_i$ oscillations is the entry of Ca^{2+} from the extracellular space [24–26]. In hepatocytes Ca^{2+} mobilizing hormones have been shown to increase the rate at which Mn^{2+} , added to the extracellular medium, quenches the intracellular fluorescence of Ca^{2+} indicators [27–30]. Assuming that Mn^{2+} utilizes the same entry pathways as Ca^{2+} this quenching could serve as a measure for the agonist-induced Ca^{2+} inflow into the cytosol [31].

The aim of this work was to investigate the effects of the anti-hyperglycaemic agents metformin and phenformin on the Ca^{2+} signal pathway in rat hepatocytes. For this purpose we have used quantitative fluorescence microscopy to examine the action of biguanides on phenylephrine-induced $[Ca^{2+}]_i$ oscillations. In addition we investigated the effects of biguanides on phenylephrine-induced Ca^{2+} movements in intact and permeabilized hepatocytes.

MATERIALS AND METHODS

Preparation and freezing of hepatocytes

Rat hepatocytes were prepared by EDTA-dissociation according to the method described by Meredith [32] with minor modifications. In brief, male Wistar rats of 200–250 g were anaesthetized by i.p. injection of 50 mg/kg Nembutal. The liver was perfused via the portal vein with Buffer A (in mM): 140 NaCl, 5 KCl, 0.8 $MgCl_2$, 1.6 Na_2HPO_4 , 0.4 KH_2PO_4 , 25 $NaHCO_3$, 2 EDTA, and an incision in the heart was made to allow outflow of the perfusate. The flow rate was adjusted to 50–60 ml/min. The buffer was kept at 37 °C and gassed with 95% O_2 /5% CO_2 . The pH was controlled by carbogen to 7.4. After about 20 min when the liver appeared softened, the perfusion was stopped. The liver was removed and washed in ice-cold Buffer B [Buffer A without EDTA and $NaHCO_3$, but containing 1 mM $CaCl_2$ /10 mM Hepes/1% BSA (pH 7.4)]. The cells were dissociated by repeated mincing, centrifuged at 30 g, and washed twice in ice-cold buffer B. After adjusting the density to 15×10^6 cells/ml, hepatocytes (about 90% viability as assessed by cell exclusion of trypan blue) were portioned by adding 0.2 ml of this suspension to a freezing vial with 0.8 ml of foetal calf serum (FCS) containing 10% dimethyl sulphoxide and then frozen in liquid nitrogen. Frozen cells were used up to 1 week after the preparation.

Fluorescence measurements in intact hepatocytes

Frozen hepatocytes were thawed at 37 °C, and washed with F-12/Hepes culture medium (containing 1.8 mM $CaCl_2$ /20 mM glucose/10 mM Hepes/2% BSA) by centrifuging twice at 30 g.

The washed hepatocytes were resuspended in F-12 culture medium supplemented with 1.8 mM $CaCl_2$ /20 mM glucose/14 mM $NaHCO_3$ /1% BSA. To allow attachment of the cells, 0.5 ml portions of the cell suspension were added to collagen R-coated culture dishes. The hepatocytes were incubated for 2 h at 37 °C in a 5% CO_2 atmosphere, humidified to saturation. Unattached cells were washed off with F-12/Hepes containing 1% BSA. The hepatocytes were then stored at 4 °C and used for up to 10 h.

Just before an experiment, the cells were either loaded by incubation with 2 μ M fura-2 acetoxymethyl ester (fura-2/AM) in F-12/Hepes culture medium supplemented with 1% BSA for 20 min at 37 °C or by diffusion of the free acid of fura-2 from a patch-pipette [33]. For this purpose the whole-cell configuration was used [34]. The pipette filling solution contained (in mM): 65 KCl, 65 potassium gluconate, 5 NaCl, 1 $MgCl_2$, 2 ATP, 10 Hepes, 0.5 fura-2/pentapotassium salt. The pH was adjusted to 7.4 with KOH. The series resistance of the pipette was typically between 2 and 6 M Ω .

Single cell fluorescence measurements of $[Ca^{2+}]_i$ were performed as previously described [19] using a Nikon-Diaphot inverted epifluorescence microscope equipped with a two-stage light amplifier and a video camera (Videscope, U.S.A.). The cells were placed in a thermostated (32 °C) chamber on the stage of the microscope. To allow the exchange of the bath medium or the addition of the agents the chamber was connected to a recirculating perfusion system. Due to the characteristics of the perfusion system the cells responded with a varying time lag of 20–50 s after addition of the respective agents. The changes of the excitation wavelengths (340 nm, 360 nm, 380 nm) were done by a computer-controlled filter wheel (Sutter Instruments/Axon Instruments, U.S.A.). $[Ca^{2+}]_i$ was estimated according to the method of Grynkiewicz et al. [35]. The quench of the fura-2 fluorescence by Mn^{2+} was measured at the calcium-insensitive excitation wavelength of 360 nm.

Fluorescence measurements in permeabilized hepatocytes

Hepatocytes were loaded with 4 μ M fluo-3 acetoxymethyl ester (fluo-3/AM) in F-12/Hepes culture medium supplemented with 1% BSA for 20 min at 37 °C. To ensure complete hydrolysis of the ester the cells were incubated for another 10 min at 37 °C in the same buffer without the dye. After this period the incubation buffer was exchanged to a cytosol-like buffer containing 100 mM KCl/20 mM NaCl/5 mM $MgCl_2$ /1 mM NaH_2PO_4 /25 mM Hepes/5 mM creatine phosphate/5 units/ml creatine kinase (pH 7.4). The cells were permeabilized by the addition of 5 μ M digitonin. The fluorescence was measured in single cells using the appropriate filter set for fluo-3 (excitation at 480 nm and emission at 520 nm). Inositol 1,4,5-trisphosphate (IP_3), heparin and biguanides were added after the cellular fluorescence declined at the same rate prior to permeabilization.

Materials

BSA, phenylephrine, glucagon, db-cAMP, forskolin and phenformin were purchased from Sigma; collagen R and F-12 culture medium from Serva; Hepes, insulin, $MnCl_2$, heparin and ionomycin from Fluka; fura-2/AM, fura-2 pentapotassium salt and fluo-3/AM from Molecular Probes; EDTA from Boehringer; FCS from Seromed/Biochrom; digitonin from Merck; metformin was a generous gift from Dr. N. Wiernsperger, Lipha (Lyon, France).

RESULTS

Inhibition of phenylephrine-induced $[Ca^{2+}]_i$ oscillations by metformin and phenformin

In order to examine possible mechanisms of action of the glucose-lowering agents metformin and phenformin, we investigated the effects of these agents on phenylephrine-induced $[Ca^{2+}]_i$ oscillations in single rat hepatocytes. Depending on the sensitivity of the isolated hepatocytes, the addition of 10–200 nM phenylephrine induced repetitive spikes of $[Ca^{2+}]_i$. Subsequent challenge by metformin or phenformin caused a partial inhibition of $[Ca^{2+}]_i$ oscillations. The inhibition was solely manifested in a decrease of the frequency of the Ca^{2+} spikes without a shape change of the single transients (Figures 1a and 1b). This inhibition could be overcome by increasing the phenylephrine concentration. Further addition of metformin or phenformin resulted again in a reduction of the frequency of the oscillations (results not shown). In fact, our results show that metformin and phenformin inhibited the phenylephrine-induced $[Ca^{2+}]_i$ oscillations in a concentration-dependent manner. The maximal effects were about a 65% reduction of the frequency of oscillations for both substances. The corresponding concentration–effect curves revealed an IC_{50} of about 0.1 mM metformin (Figure 1c). However, for the therapeutically more potent biguanide phenformin the half-effective concentration was two orders of magnitude lower than that of metformin, i.e. an IC_{50} of about 1 μ M (Figure 1d).

Alteration of the biguanide action by insulin and glucagon

In a previous study from our laboratory [23] it was demonstrated that phenylephrine-induced $[Ca^{2+}]_i$ oscillations could be nega-

Table 1 Enhanced inhibition of agonist-induced $[Ca^{2+}]_i$ oscillations by subsequent application of biguanides and insulin

The reduction of the frequency of phenylephrine-induced $[Ca^{2+}]_i$ oscillations by biguanides and insulin and the simultaneous application of the agents is shown in the Table. The values denote the mean and S.E.M. for the respective agents.

	Insulin (10 nM)	Metformin (0.1 mM)	Phenformin (1 μ M)	Insulin and metformin	Insulin and phenformin
Inhibition of the frequency (%)	35 $\pm$ 4.8	30 $\pm$ 2.5	32 $\pm$ 2.7	58 $\pm$ 5.6	76 $\pm$ 5.8
<i>n</i>	28	29	13	14	21

tively and positively modulated by insulin and glucagon, respectively. Could biguanides enhance the inhibitory action of insulin on hormone-induced $[Ca^{2+}]_i$ oscillations? After inducing $[Ca^{2+}]_i$ oscillations with 10–20 nM phenylephrine the addition of insulin (most effective dose 10 nM), metformin (0.1 mM) or phenformin (1 μ M) decreased the frequency of the Ca^{2+} transients. Furthermore the subsequent application of insulin and biguanides resulted in an enhanced frequency reduction of the oscillations (Table 1). The degree of the additional inhibition was independent of the sequence in which these agents were added. Again the decrease of the frequency caused by biguanides and/or insulin could be antagonized by a subsequent increase of the phenylephrine concentration.

In contrast, glucagon, the physiological counterpart of insulin, exerts a positive modulatory influence on agonist-induced $[Ca^{2+}]_i$

Figure 1 Inhibition of phenylephrine-induced $[Ca^{2+}]_i$ oscillations by biguanides in single rat hepatocytes

Addition of the α_1 -adrenergic agonist phenylephrine caused sustained $[Ca^{2+}]_i$ oscillations. Subsequent addition of 0.5 mM metformin (a) and 3 μ M phenformin (b) resulted in a decrease of the frequency of $[Ca^{2+}]_i$ oscillations. The addition of the different agents to the bath solution is indicated by the respective bars. The traces represent a typical experiment out of 12 and 11 individual experiments with metformin and phenformin, respectively. Performing similar experiments with different concentrations of the biguanides led to the concentration–effect curves shown in (c) and (d). Bars denote S.E.M. and the number of experiments is given in parentheses.

Table 2 Overview of the effect of elevated cytosolic [cAMP] on the partial inhibition of phenylephrine-induced $[Ca^{2+}]_i$ oscillations by biguanides

This Table shows the number of experiments where the antagonistic effect of elevated $[cAMP]_{cyt}$ by different stimuli on the partial inhibition of $[Ca^{2+}]_i$ oscillations has been observed. The numbers in parentheses indicate the total number of experiments for the corresponding combinations.

Partial inhibition by	Restoration of oscillations by		
	Glucagon (10–100 nM)	Forskolin (1–10 μ M)	db-cAMP (2–5 μ M)
Metformin	11 (11)	7 (9)	4 (4)
Phenformin	9 (11)	7 (9)	7 (9)

oscillations via the cAMP second messenger system [23]. Therefore we investigated whether activation of the cAMP signalling pathway could counteract the inhibitory action of biguanides on phenylephrine-induced $[Ca^{2+}]_i$ oscillations. Indeed the addition of 10–100 nM glucagon (physiological activator of the adenylate cyclase), 1–10 μ M forskolin (direct activator of the adenylate cyclase) or 2–5 μ M db-cAMP (a membrane-permeable cAMP analogue) elevated the frequency of $[Ca^{2+}]_i$ oscillations after the inhibition with metformin or phenformin. Even the enhanced inhibition of insulin applied together with biguanides could be antagonized by increasing the cytosolic (cAMP) ($[cAMP]_{cyt}$) with the different agonists. The experiments dealing with the antagonism of glucagon, forskolin and db-cAMP on the inhibi-

tion of phenylephrine-induced $[Ca^{2+}]_i$ oscillations by biguanides are summarized in Table 2.

The effect of metformin and phenformin on the phenylephrine-induced $MnCl_2$ quench

Since sustained $[Ca^{2+}]_i$ oscillations require Ca^{2+} influx from the extracellular space, we investigated whether biguanides affect phenylephrine-induced Ca^{2+} entry by measuring the Mn^{2+} quench of the cellular fura-2 fluorescence. Figure 2 shows the response of single fura-2/AM-loaded hepatocytes to the replacement of extracellular Ca^{2+} with 1 mM Mn^{2+} and the subsequent addition of phenylephrine and metformin. Addition of 1 mM Mn^{2+} resulted in a rapid quench of cellular fluorescence measured at the isosbestic excitation wavelength of 360 nm. Following this initial response, the fluorescence declined at the rate recorded prior to the addition of Mn^{2+} . Subsequent addition of phenylephrine (2 μ M) caused a second rapid quench of cellular fura-2 (see upper trace in Figure 2a). Note that, however, we had to use elevated phenylephrine concentrations (up to 2 μ M) in order to observe significant effects. Addition of metformin (3 mM) (Figure 2b, upper trace) inhibited the phenylephrine-induced quench of cellular fluorescence. The lower traces in Figure 2 show the simultaneous estimation of $[Ca^{2+}]_i$ via the fluorescence ratio measured at 340 nm/380 nm. Surprisingly the addition of 1 mM Mn^{2+} caused an increase of this ratio, reaching a new steady-state level. The subsequent addition of phenylephrine resulted in a decrease of the ratio even below the initially measured resting level (Figure 2a, lower trace). High concen-

Figure 2 Effect of phenylephrine and metformin on Mn^{2+} quench and fluorescence ratio of cellular fura-2 in single fura-2/AM-loaded hepatocytes

Rat hepatocytes were loaded with fura-2 by incubation with the acetoxymethyl ester. The upper traces show the time course of cellular fluorescence measured at the Ca^{2+} -insensitive excitation wavelength of 360 nm. As an estimation of the $[Ca^{2+}]_i$ the fluorescence ratio 340 nm/380 nm was measured simultaneously. (a) The response of these parameters to the sequential addition of Mn^{2+} and phenylephrine. (b) The effect of subsequent application of metformin on the phenylephrine-induced changes. (c) Inhibition of the phenylephrine-induced changes by prior addition of metformin are reversed after wash-out of the biguanide. The presence of the different agents in the bath solutions is indicated by the respective bars. The traces shown in (a), (b) and (c) are typical findings out of 35, 20 and 15 experiments, respectively.

Figure 3 Effect of phenylephrine and biguanides on Mn^{2+} quench of cellular fura-2 in single hepatocytes loaded with fura-2/pentapotassium salt via a patch pipette

Hepatocytes were loaded with fura-2/pentapotassium salt via a patch pipette. The cellular fluorescence was measured at the excitation wavelength of 360 nm, as shown in the upper traces in (a) and (b). In the same cells $[Ca^{2+}]_i$ was estimated from the fluorescence ratio 340 nm/380 nm (lower traces in a and b). Addition of 1 mM Mn^{2+} caused a monotonic quenching of cellular fluorescence (upper traces) whereas no change in the fluorescence ratio occurred (lower traces). Subsequent addition of phenylephrine caused no steeper decline of the quench rate. The oscillations of the 360 nm signal after addition of phenylephrine are presumably due to a cross-talk resulting from the wavelength (360 nm) being slightly longer than the isosbestic wavelength in the cytosol (see also ref. 44). Addition of metformin (a) and phenformin (b) did not inhibit the Mn^{2+} quench, but reduced the frequency of the oscillations. The records in (a) and (b) were confirmed in 5 and 4 different single cell measurements, respectively.

trations of metformin (Figure 2b, lower trace) inhibited the hormone-induced decrease of the fluorescence ratio. As a control we added metformin prior to phenylephrine. Figure 2(c) shows that metformin (3 mM) completely abolished the response to the subsequent addition of phenylephrine. After wash-out of metformin, phenylephrine induced an increase of the quench rate (Figure 2c, upper trace) and a decrease of the fluorescence ratio (Figure 2c, lower trace). Similar results were obtained by using 30 μ M phenformin instead of metformin (results not shown). Is the Mn^{2+} -induced increase of the fluorescence ratio an artefact due to the quench of the fura-2 signal by Mn^{2+} ? To test this alternative we loaded single hepatocytes with fura-2/pentapotassium salt via a patch pipette. As shown in the upper traces of Figure 3 the replacement of Ca^{2+} by Mn^{2+} caused a slower and sustained decrease of the cellular fluorescence. In contrast to the experiments performed with AM-loaded cells, with Mn^{2+} alone the fluorescence signal at 360 nm vanished almost completely after prolonged recordings (results not shown). Subsequent addition of 2 μ M phenylephrine, 3 mM metformin or 30 μ M phenformin during the still on-going fluorescence quench caused no significant change of the initial quench rate (Figure 3, upper traces). Even by increasing the phenylephrine concentration to 4 μ M or by addition of up to 100 nM vasopressin we still did not observe an agonist-induced Mn^{2+} quench (results not shown). The lower traces in Figure 3 show the simultaneous estimation of the cellular $[Ca^{2+}]_i$. In contrast to AM-loaded cells the addition of Mn^{2+} had no effect on the cellular $[Ca^{2+}]_i$, indicating that the increase of the ratio in AM-loaded cells was not due to an artefact based on the quench of the fura-2 signal by Mn^{2+} . Note

that after addition of phenylephrine we recorded an oscillatory $[Ca^{2+}]_i$, sensed by the remaining unquenched fura-2 in the cytosol.

Effect of biguanides on the IP_3 -induced release of Ca^{2+} from intracellular stores in permeabilized hepatocytes

A reasonable explanation of these observations is that in hepatocytes loaded with fura-2/AM a distribution of the indicator dye occurs into intracellular compartments. To further investigate this possibility more directly, we loaded single rat hepatocytes with the Ca^{2+} indicator fluo-3/AM. Owing to the lower affinity of fluo-3 for Ca^{2+} , as compared with fura-2, we had the possibility of measuring $[Ca^{2+}]_i$ changes even at the presumably high Ca^{2+} concentrations inside the intracellular Ca^{2+} stores. After permeabilization of single cells with 5 μ M digitonin the cellular fluorescence declined to about one-third of the initial value. The relatively high value of the remaining fluorescence supported the idea that fluo-3/AM is sequestered into intracellular compartments. An assumption underlying this interpretation is that the concentration of digitonin used only permeabilizes the plasma membrane without affecting the membranes of the intracellular stores. The addition of 0.2 μ M IP_3 resulted in a rapid partial decrease of the remaining fluorescence, indicating a decrease of $[Ca^{2+}]_i$ in IP_3 -sensitive intracellular Ca^{2+} stores (Figure 4a). Moreover, adding increasing IP_3 concentrations in a stepwise fashion resulted in further reductions of the fluo-3 signal, indicative of a so called 'quantal' Ca^{2+} release observed in permeabilized cells (results not shown; compare with ref. 36). The subsequent addition of the Ca^{2+} ionophore ionomycin and $MnCl_2$

Figure 4 IP_3 -induced release of Ca^{2+} from intracellular stores measured by the fluorescence change of compartmentalized fluo-3 in single permeabilized fluo-3/AM-loaded hepatocytes

The main emphasis of Figure (a) consists of documenting the permeabilization protocol. Hepatocytes were loaded with fluo-3/AM and after exchange of the bath medium to the permeabilization buffer they were permeabilized by addition of $5 \mu\text{M}$ digitonin, as indicated. The subsequent addition of $0.2 \mu\text{M}$ IP_3 , $2 \mu\text{M}$ ionomycin and 1 mM Mn^{2+} are indicated by the respective arrows. For a better comparison of the individual experiments the fluorescence intensity was normalized to the fluorescence at the time of IP_3 addition ($t = 0 \text{ min}$). As a control, heparin was added 2 min before IP_3 . As shown in (b) heparin treatment (dotted line) inhibited the IP_3 -induced release of Ca^{2+} (continuous line) effectively. The results shown in (a) and (b) are averages from three individual cells from the same preparation and are representative of findings in 25 and 9 single cell measurements, respectively.

Figure 5 Effect of biguanides on IP_3 -induced Ca^{2+} release in single permeabilized hepatocytes loaded with fluo-3/AM

Permeabilization of hepatocytes loaded with fluo-3/AM by $5 \mu\text{M}$ digitonin resulted in a decrease of cellular fluorescence to a new steady-state level. Only the remaining part of the traces after permeabilization are shown (c.f. Figure 5). The addition of $0.2 \mu\text{M}$ IP_3 resulted in a decrease of the normalized remaining fluorescence (solid line). Prior addition of 1 mM metformin (broken line) or $10 \mu\text{M}$ phenformin (dotted line) reduced the rate and extent of the IP_3 -induced decline of the remaining fluorescence. The results shown are averaged from four single cell measurements in the same preparation and were confirmed in 5 other preparations with a total of 30 single cells measured.

caused the release of Ca^{2+} from IP_3 -insensitive compartments and the complete quench of the cellular fluorescence, respectively. As a control we added $20 \mu\text{g/ml}$ of the IP_3 receptor antagonist

heparin to permeabilized hepatocytes prior to the addition of IP_3 . As shown in Figure 4(b), heparin effectively blocked the IP_3 -induced decrease of the fluorescence. Could the inhibitory effect of biguanides on $[\text{Ca}^{2+}]_i$ oscillations be explained by a direct interaction with the IP_3 receptor of intracellular Ca^{2+} stores? To test this possibility we investigated the effect of biguanides on the IP_3 -induced release of Ca^{2+} in permeabilized single rat hepatocytes. As shown in Figure 5 the addition of $0.2 \mu\text{M}$ IP_3 caused the release of Ca^{2+} from intracellular stores (solid line). In cells from the same preparation the addition of 1 mM metformin (broken line) and $10 \mu\text{M}$ phenformin (dotted line) prior to the application of IP_3 reduced the rate and the amount of Ca^{2+} released from intracellular stores. Within the concentration range used, the biguanides added alone had no effect on the cellular fluorescence in permeabilized hepatocytes (results not shown).

DISCUSSION

Concerning the glucose-lowering effects of the biguanides metformin and phenformin thus far there is evidence that they are mainly mediated via an altered glucose metabolism in peripheral tissues [1]. In this study we have investigated whether biguanides can modulate $[\text{Ca}^{2+}]_i$ oscillations in isolated rat hepatocytes induced by the glycogenolytic agonist phenylephrine. We found that indeed the addition of the biguanides elicited a concentration-dependent reduction of the frequency of the oscillations. In agreement with the documented 100-fold higher therapeutic potency of phenformin as compared with metformin [37] we found the IC_{50} for phenformin to be two orders of magnitude lower than that of metformin. However, the concentrations required were higher than the therapeutically effective concentrations used in the treatment of NIDDM [8]. There is evidence that biguanides require the presence of insulin for their glucose lowering action *in vivo* [7,8,16]. The significantly increased lowering of the frequency by simultaneous application of insulin and biguanides compared with the effects observed when these agents were added alone, speaks in favour of the enhanced metabolic effects of biguanides by insulin.

In rat liver membranes of lean and obese Zucker rats it was shown that metformin decreased the ability of glucagon to stimulate the adenylate cyclase activity [38]. Do biguanides influence the agonist-induced $[\text{Ca}^{2+}]_i$ oscillations predominantly by altering the cAMP signalling pathway? It has been shown that activation of c-AMP-dependent protein kinase (PKA) via increasing $[\text{cAMP}]_{\text{cyt}}$ potentiates IP_3 -induced Ca^{2+} mobilization in permeabilized rat hepatocytes [39,40] and increases the frequency of phenylephrine-induced $[\text{Ca}^{2+}]_i$ oscillations [23,41]. Furthermore, Somogyi et al. [23] have found that adenosine-3',5'-cyclic monophosphorothionate R_p -diastereomer, a membrane permeant inhibitor of PKA, could suppress phenylephrine-induced $[\text{Ca}^{2+}]_i$ oscillations. However, in the absence of cAMP-linked stimuli this inhibitory effect was barely detectable. Thus it appears that the cAMP pathway is not necessary for, but acts as a positive modulator in the generation of, the Ca^{2+} response. Hence it seems unlikely that the inhibitory action of biguanides on $[\text{Ca}^{2+}]_i$ oscillations is mainly mediated via an alteration of the cAMP signalling pathway.

To further narrow down the possible site of action of the biguanides, we studied their effects on the phenylephrine-induced Mn^{2+} quench of the fura-2 fluorescence in intact hepatocytes either loaded with fura-2/AM or the free acid form of the dye. First of all these experiments clearly demonstrated that hepatocytes loaded with fura-2/AM compartmentalize a significant amount of the dye into intracellular organelles. This is further

supported by the fact that in cells loaded with the membrane-impermeable fura-2/pentapotassium salt the addition of Mn^{2+} caused no increase of the fluorescence ratio measured at 340/380 nm compared with the cells loaded with fura-2/AM. Glennon et al. [30] came to a similar conclusion to explain the failure of the endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin to induce a Mn^{2+} influx into hepatocytes loaded with fura-2/AM [28,29] which was not the case in cells micro-injected with fura-2/pentapotassium salt. In AM-loaded cells the monotonic decrease of the ratio and the second quench phase after addition of phenylephrine could then be interpreted as an efflux of Ca^{2+} out of and an influx of cytosolic Mn^{2+} into the stores, respectively, due to the opening of the IP_3 -operated Ca^{2+} channel. In this context it should be noted that this response could only be observed if we used 10–100 times higher phenylephrine concentrations compared with the concentrations required to induce $[Ca^{2+}]_i$ oscillations. At this hormone concentration hepatocytes are overstimulated, meaning that the IP_3 receptor is maximally activated [19]. This indicates that a complete and sustained Ca^{2+} release from intracellular stores is necessary to induce the observed response.

As a consequence, in hepatocytes loaded with fura-2/AM, the phenylephrine-induced Mn^{2+} quench and the decrease of the fluorescence ratio may primarily be used as an indicator of the Ca^{2+} flux across intracellular membranes. Hence, the inhibitory effect of biguanides indicates an alteration of the phenylephrine-induced Ca^{2+} release from intracellular Ca^{2+} stores. This is further supported by our findings obtained from experiments with permeabilized hepatocytes loaded with fluo-3/AM. There we observed that the IP_3 -induced Ca^{2+} release from intracellular stores could be partially inhibited by prior addition of metformin or phenformin. In contrast to Renard-Rooney et al. [42] we observed a quantal Ca^{2+} release in single permeabilized and attached hepatocytes. These authors could show that permeabilization by digitonin caused a vesicularization of the endoplasmic reticulum in cells held in suspension, but not in attached cells. This finding could explain why only in unattached cells is the so called 'quantal' Ca^{2+} release observed. Our results obtained in single, attached hepatocytes might be due to the different isolation protocol of the cells and the use of collagen R instead of polylysine for attachment. Therefore it cannot be excluded that in our preparation a vesicularization of the endoplasmic reticulum might occur after permeabilization.

Our results strongly suggest that the anti-diabetic drugs of the biguanide type inhibit phenylephrine-induced $[Ca^{2+}]_i$ oscillations by a direct interaction with the IP_3 receptor/ Ca^{2+} channel complex thereby reducing the IP_3 -induced Ca^{2+} release. Furthermore, we could show that the normally unwanted compartmentalization of fluorescent Ca^{2+} indicators into intracellular structures opens up the possibility of estimating the $[Ca^{2+}]_i$ in IP_3 -sensitive stores in intact cells. By using a similar approach based on the indicator dye Mag-fura-2/AM, Hofer and Machen [43] have already shown that the technique is indeed a suitable tool for *in situ* measurements of hormone-induced changes of the Ca^{2+} content of intracellular stores. Furthermore our findings underline once more the importance of double-checking the results by using the two different loading procedures since otherwise a clear-cut interpretation of changes in Ca^{2+} fluxes across the different cellular and intracellular membranes is not warranted [30].

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