Free Rad. Res., Vol. 33, pp. 507-516
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Oxidative Stress Increases Potassium Efflux From Pancreatic Islets By Depletion Of Intracellular Calcium Stores

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Accepted for publication by Prof. H. Sies

(Received 09 December 1999; In revised form 28 March 2000)

Oxidative stress to B-cells is thought to be of relevance in declining B-cell function and in the process of B-cell destruction. In other tissues including heart, brain and liver, oxidative stress has been shown to elevate the intracellular free calcium concentration and to provoke potassium efflux. We studied the effect of oxidative stress on Ca^{2+} and K^+ (Rb^+) outflow from pancreatic islets using the thiol oxidants DIP and BuOOH. Both compounds reversibly increased $^{86}Rb^+$ efflux in the presence of 3 and 16.7 mmol/l glucose. Stimulation of $^{86}Rb^+$ efflux was also evident in the absence of calcium. DIP evoked release of $^{45}Ca^{2+}$ from the pancreatic islets both in the presence or absence of extracellular calcium. Employing inhibitors of the calcium-activated potassium channel (K_{Ca}) and the high conductance K^+-channel (BK_{Ca}), the effect of DIP on $^{86}Rb^+$ efflux was slightly diminished. Tolbutamide had no effect on $^{86}Rb^+$ efflux in the presence of DIP. On the other hand thapsigargin, a blocker of the Ca^{2+}-ATPase of the endoplasmic reticulum, completely suppressed the DIP-mediated $^{86}Rb^+$ outflow. The data suggest that thiol oxidant-induced potassium efflux from pancreatic islets is mainly mediated through liberation of intracellular calcium and subsequent stimulation of calcium-activated potassium efflux.

Keywords: Oxidative stress; pancreatic islets; BuOOH; DIP; potassium efflux; thapsigargin

Abbreviations: DIP, diazene dicarboxylic acid *bis* (N'-methylpiperazide); DIP^{2+}, *-bis* N-methyliodide; BuOOH, tert-butylhydroperoxide; TEA, tetraethylammonium

INTRODUCTION

The redox state of GSH has been suggested to be of relevance in the mechanism of insulin secretion in pancreatic islets as previously reviewed[1]. GSH is important in a variety of reactions including reductive processes, protection of cells against oxidative stress, free oxygen species and other toxic compounds of endogenous and exogenous origin. Two mechanisms have been described by which oxidants alter the function of ion channels in liver and cardiac tissue. One appears to be the incresase in intracellular free Ca^{2+} concentration[2], the second is due to an enhanced potassium efflux along ATP-sensitive potassium channels[3,4] which is considered to be a valuable approach in protecting the myocardium against injury[5]. In pancreatic islets the closure of K^+ATP channels in response to a rise in the intracellular ATP/ADP ratio

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causes β-cell depolarization and subsequent opening of voltage-sensitive Ca^{2+} channels. The resulting Ca^{2+} -influx triggers insulin secretion.

Pancreatic islets are extremely sensitive to oxidative stress because the activity of the GSH peroxidase as well as the GSH/GSSG ratio are low compared to other tissues[6]. There is evidence that the redox state of intracellular thiols and pyridine nucleotides is related to calcium uptake and insulin secretion. A decrease of the GSH/GSSG ratio by thiol reagents penetrating into islet cells causes inhibition of calcium uptake and insulin secretion. Therefore, oxidative stress to B-cells may be of relevance during the process of B-cell destruction in the developement of type I diabetes[7].

We therefore investigated the influence of the membrane permeable sulfhydryl (SH) group-oxidizing agent diazene dicarboxylic acid *bis* (N'-methylpiperazide) (DIP), its nonpenetrating – and therefore only extracellular acting analog – diazene dicarboxylic acid bis-(N'-methylpiperazide)$^{2+}$ (DIP^{2+}) and the organic peroxide tert-butylhydroperoxide (BuOOH) on K^+ and Ca^{2+} efflux from pancreatic islets of the rat. To define the mechanism behind the altered K^+ and Ca^{2+} channel currents essential to normal β-cell function we used a variety of K^+ channel blockers and thapsigargin as an ER-Ca^{2+}-ATPase inhibitor. The ATP/ADP ratio in presence of oxidants in pancreatic islets was determined too.

MATERIALS AND METHODS

Chemicals

Collagenase (0.8 PZU/mg) was obtained from Serva Feinbiochemika, Heidelberg, Germany; bovine serum albumin was purchased from Sigma Chemical & Co., St. Louis, USA; $^{86}RbCl$ and $^{45}CaCl_2$ were supplied by Amersham-Buchler, Braunschweig, Germany; Thapsigargin, tetraethylammonium (TEA) and penitrem A were purchased from Sigma, St. Louis, USA. Tert-butylhydroperoxide (BuOOH) was obtained from Sigma-Aldrich, Deisenhofen, Germany. Diazene dicarboxylic acid bis-(N'-methylpiperazide) (DIP) and bis-N'-methyliodide (DIP^{2+}) were synthesized according to Kosower and Kanety-Londner[8]. Firefly luciferase, D-luciferin (synthetic, free acid), ATP-Sulfurylase and sodium molybdate were from Sigma, St. Louis, USA. Creatine phosphate, creatine kinase, ADP, ATP and GMP were obtained from LaRoche Biochemicals, Mannheim, Germany. All other chemicals and reagents of analytical grade were obtained from E. Merck, Darmstadt, Germany.

Isolation and Incubation of rat pancreatic Islets

For the preperation of islets, Wistar rats (200–300 g) of both sexes obtained from a local strain were used. Pancreatic islets were prepared by collagenase technique as reported earlier[9]. The ionic composition of the medium used was as follows (mmol/l): NaCl 122, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.1, $NaHCO_3$ 20. The buffer was gassed with 95 % O_2 – 5 % CO_2 and supplemented with 0.5 % bovine serum albumin and 5.6 mmol/l glucose. The pH was adjusted to 7.4 in all experiments. $NaHCO_3$ was replaced by 10 mmol/l HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and gassed with ambient air. In the case of calcium-deprived medium $CaCl_2$ was replaced by $MgCl_2$.

Measurement of $^{86}Rb^+$ and $^{45}Ca^{2+}$ Efflux from perifused rat pancreatic Islets

Groups of fifty islets were incubated for 90 min in Krebs-Ringer-HEPES buffer containing $^{86}RbCl$ and glucose 5.6 mmol/l. After washing the islets were placed into perifusion chambers (0.3 ml). The perifusate was conveyed at 37 °C and at a flow rate of 1.2 ml/min. After an equilibration period of 20 min, $^{86}Rb^+$ appearing in the

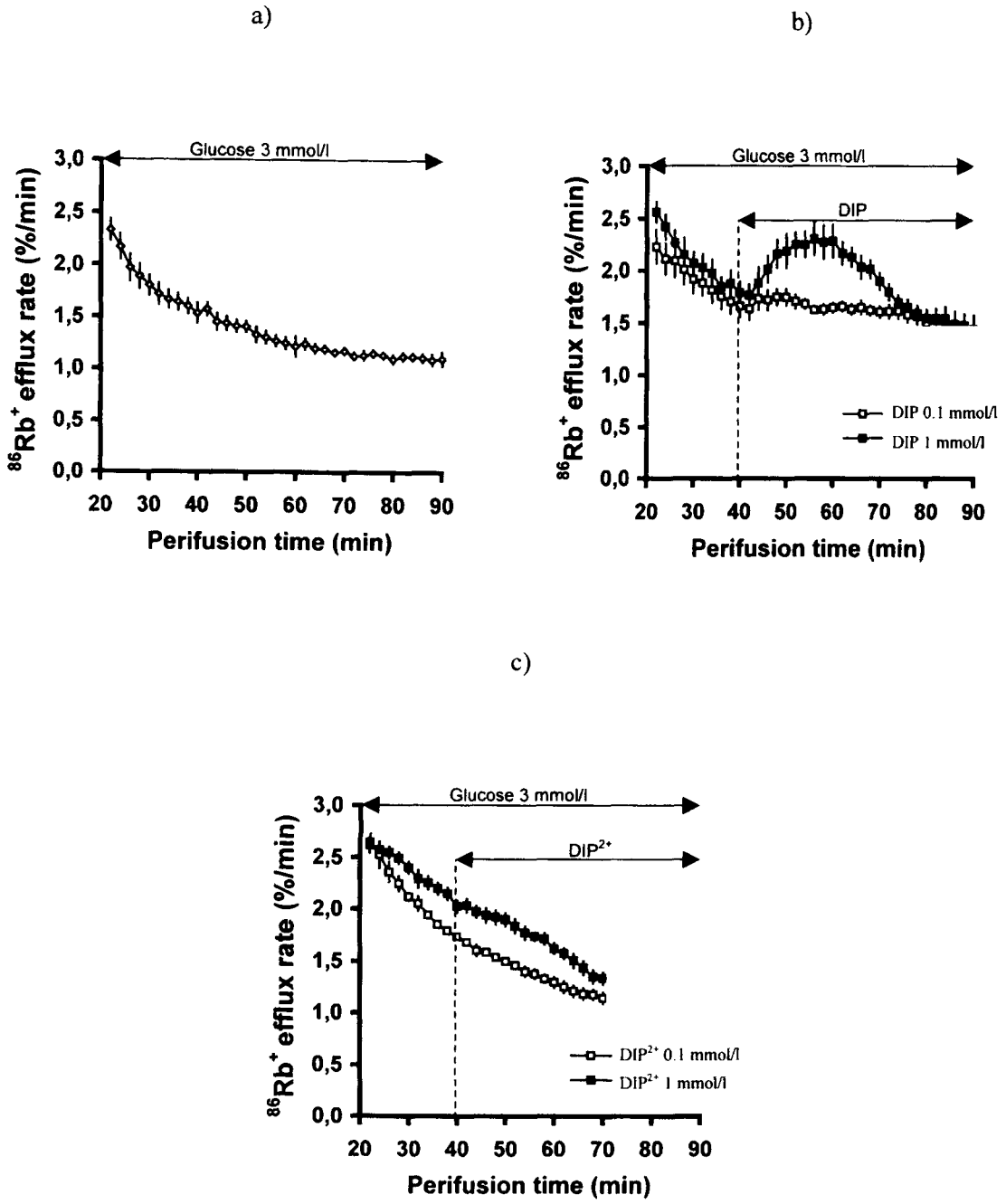

FIGURE 1 Effect of the thiol oxidant diazene dicarboxylic acid *bis* (N-methylpiperazide) (DIP, Fig. 1b) and *bis* N-methyliodide (DIP^{2+}, Fig. 1c) on $^{86}Rb^{+}$ efflux rate from perifused rat pancreatic islets. During the interval 40–90 min, DIP or DIP^{2+} (0.1 mmol/l or 1.0 mmol/l) was added to the perifusion medium. Glucose concentration was 3 mmol/l throughout the experiment (Fig. a – c). Mean values ± SEM, n = 3

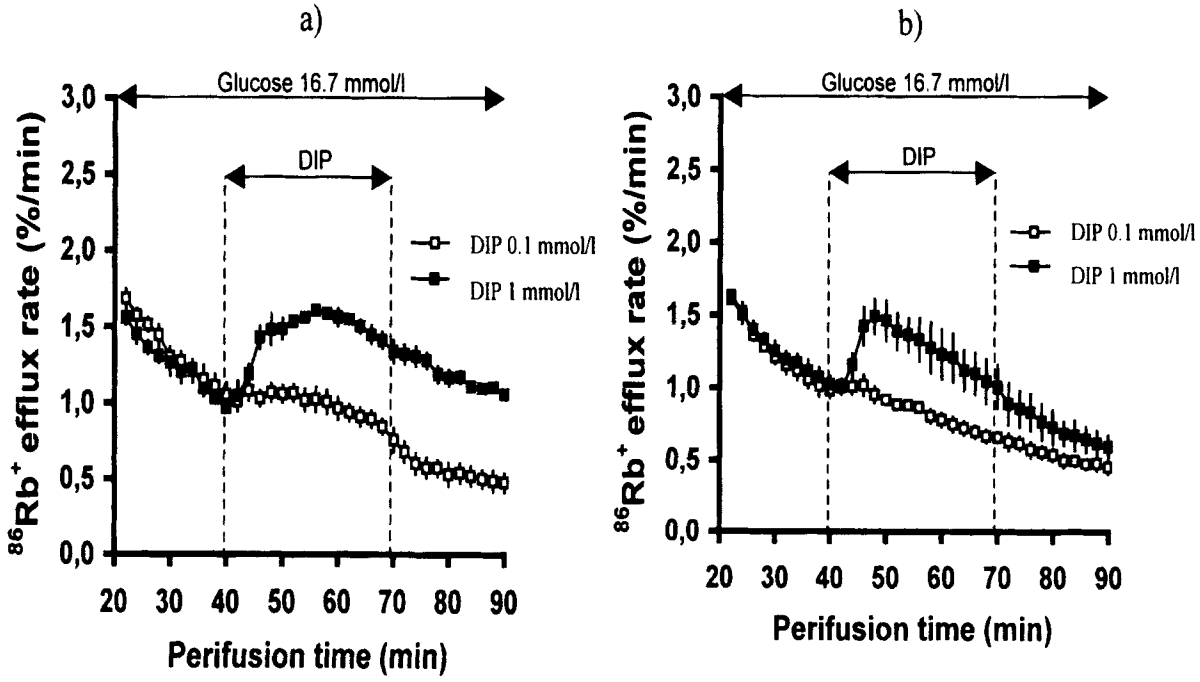

FIGURE 2 Effect of the thiol oxidant diazene dicarboxylic acid *bis* (N-methylpiperazide) (DIP) on $^{86}Rb^+$ efflux rate from perifused rat pancreatic islets. DIP (0.1 mmol/l or 1 mmol/l) was added from 40–70 min. Glucose concentration was 16.7 mmol/l throughout the experiment. Fig. 2a shows the efflux in calcium-containing medium, the right panel (b) shows the effect of DIP in calcium-free medium. Mean values ± SEM, n = 3

effluent fractions (collected at 2 min intervals) was determined. The fractional efflux of radioactivity was calculated as the percentage release rate of instantaneous islet content of $^{86}Rb^+$ at each time point. During the interval indicated in the figure, DIP, DIP^{2+} or BuOOH (0.1 mmol/l – 1 mmol/l) were added to the perifusion medium.

For $^{45}Ca^{2+}$ efflux measurements, 80 islets were incubated in Krebs-Ringer-HEPES buffer containing $^{45}CaCl_2$ and glucose 16.7 mmol/l. The equilibration period took 40 min and the fractions were collected at 1 min intervals.

Assay of the ATP/ADP ratio from rat pancreatic Islets

The determination of ATP and ADP was performed according to the procedure described by Schultz et al.[10]. Twenty rat islets were incubated in KRH buffer supplemented with 3 mmol/l glucose. The incubation with the thiol oxidants was stopped by removing the medium and lysis of islets in NaOH (0.2 mmol/l). The lysate was stored at –20 °C until use. To assay ATP, an aliquot was incubated with a buffer containing (mmol/l): Hepes 50, EGTA 2, $MgSO_4$ 10, KCl 20, BSA 0.5 %, firefly luciferase (0.1 µmol/l) and D-luciferin (100 µmol/l) and adjusted to pH 7.6 with KOH. ATP content was calculated from a standard curve. ADP was determined from a second aliquot. Initially, ATP was degraded to AMP by 30 min incubation with buffer containing (mmol/l): Tris 50, $MgCl_2$ 5, $NaMoO_4$ 10, GMP 15 and sulfurylase (30 µU/ml). pH was adjusted to 8.0 with HCl. At the end, sulfurylase was deactivated by a 5 min incubation in boiling water. In a second step, ADP was converted to

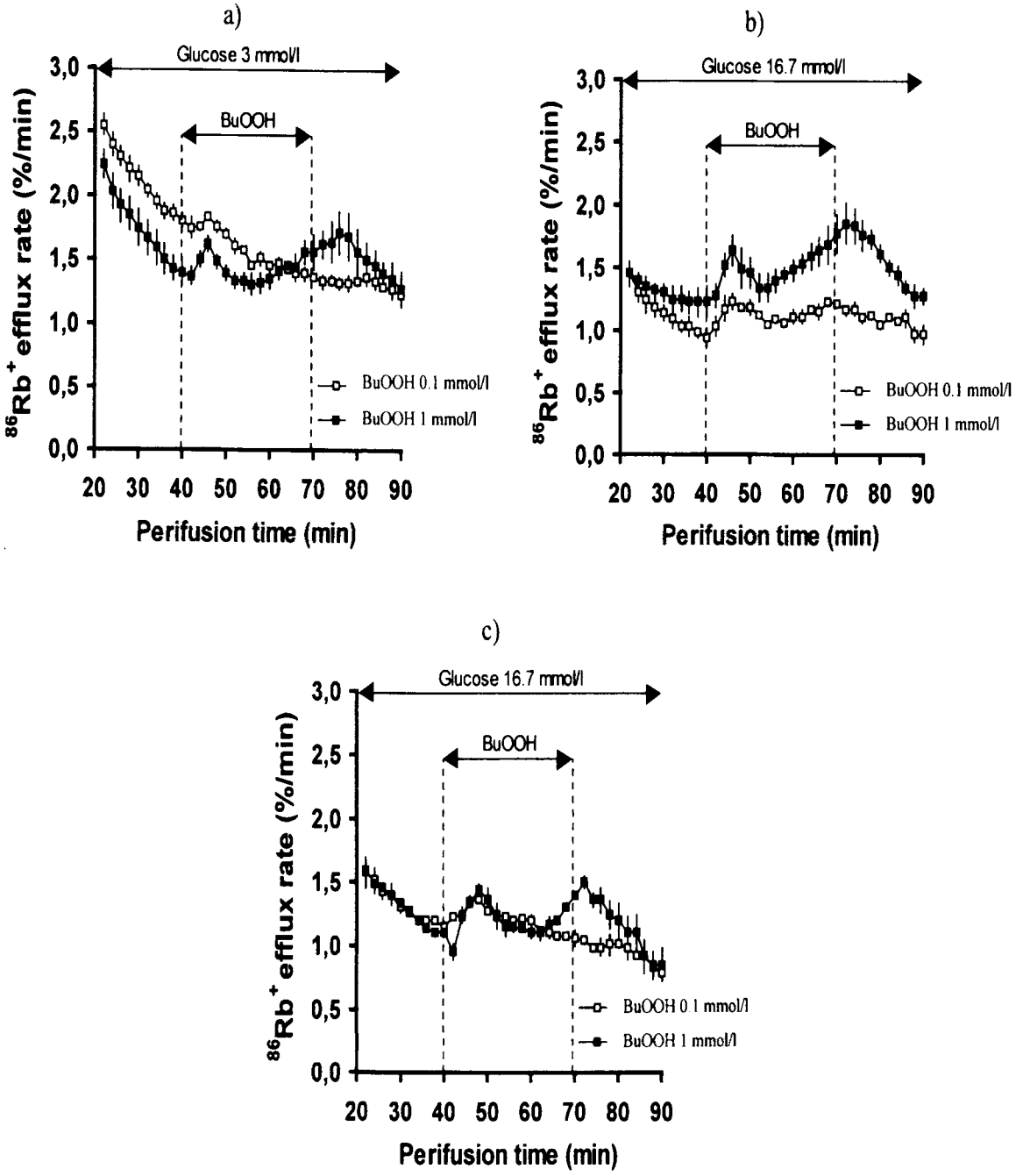

FIGURE 3 Effect of tert-butylhydroperoxide (BuOOH) on $^{86}Rb^+$ efflux rate from perifused rat pancreatic islets. During the interval 40–70 min, BuOOH (0.1 mmol/l or 1 mmol/l) was added to the perifusion medium. The islets were perifused throughout in the presence (Fig. 3a+b) or absence (Fig. 3c) of extracellular Ca^{2+}. Glucose concentration was 3 mmol/l or 16.7 mmol/l throughout the experiment. Mean values ± SEM, n = 3

ATP using a buffer consisting of (mmol/l): Tris 50, $MgCl_2$ 5, KCl 40, creatine phosphate 2 and creatine kinase (10 μU/ml). After 30 min of incubation at room temperature KRH buffer was added. ATP was measured as described above and the content was determined from a standard curve prepared with ADP standards processed in parallel.

Statistics

The significance of differences between groups was determined by analysis of variance (ANOVA) followed by Newman-Keuls test. Data are presented as mean ± SEM, n representing the number of independent experiments.

RESULTS

Effects of DIP and DIP^{2+} on $^{86}Rb^+$ efflux rate from pancreatic islets

Addition of DIP 0.1 mmol/l did not affect the $^{86}Rb^+$ efflux rate in the presence of 3 mmol/l glucose. However, at higher concentration (1 mmol/l), DIP revealed a sustained increase in $^{86}Rb^+$ efflux rate (Fig. 1b). At 1 mmol/l DIP the maximum effect appeared between 15 and 20 minutes after addition of the thiol oxidant, followed by a decline of the $^{86}Rb^+$ outflow. The addition of DIP^{2+} (0.1 – 1 mmol/l) to islets did not modify the $^{86}Rb^+$ efflux rate at any concentration tested (Fig. 1c). When the perifusion medium was enriched with a stimulating (16.7 mmol/l) glucose concentration, the islets exhibited a similar change in the $^{86}Rb^+$ efflux rate in response to DIP compared with 3 mmol/l glucose (Fig. 2a). Under these conditions, DIP increased the $^{86}Rb^+$ efflux too when calcium was removed from the perifusion medium (Fig. 2b) although this effect was less pronounced.

Effects of BuOOH on $^{86}Rb^+$ efflux rate from pancreatic islets

Using BuOOH as an oxidant in a Ca^{2+}-containing medium a concentration-dependent biphasic increase in $^{86}Rb^+$ outflow was seen (Fig. 3a). The magnitude of this effect was identical whether the perifusion medium contained 3 or 16.7 mmol/l of glucose (Fig. 3b). In the absence of calcium (Fig. 3c), the increase was nearly similar as in the presence of calcium. The change in $^{86}Rb^+$ efflux rate after removal of the drug reflects relief from the stimulating effect of BuOOH.

Effects of K^+-channel blockers on the $^{86}Rb^+$ efflux rate in calcium-free medium

The effects of a variety of inhibitors of potassium channels (tolbutamide: K^+ ATP channel, penitrem A: voltage and calcium dependent potassium channel, tetraethylammonium: calcium dependent potassium channel) at 3 mmol/l glucose in the absence of Ca^{2+} was tested. None of these inhibitors was able to completely abolish the $^{86}Rb^+$ efflux caused by DIP (Fig. 4). However, penitrem A and tetraethylammonium (TEA) led to a slightly depressed efflux rate with time whereas tolbutamide exhibited no effect at all. Similar results were obtained using BuOOH as an oxidant (data not shown).

Effects of a preincubation with thapsigargin on the $^{86}Rb^+$ efflux rate in calcium-free medium

Thapsigargin is an inhibitor of the endoplasmic reticulum-Ca^{2+} ATPase. When pancreatic islets were preincubated with thapsigargin (2 μmol/l) for 30 min before addition of DIP, $^{86}Rb^+$ efflux was abolished in response to the oxidant. (Fig. 5). BuOOH also failed to affect the $^{86}Rb^+$ efflux rate from islets exposed to thapsigargin (data not shown).

Effects of DIP on $^{45}Ca^{2+}$ efflux rate from islets in the presence or absence of calcium

Whether the medium contained or was deprived of extracellular Ca^{2+}, DIP caused a similar increase in the $^{45}Ca^{2+}$ efflux rate (data not shown).

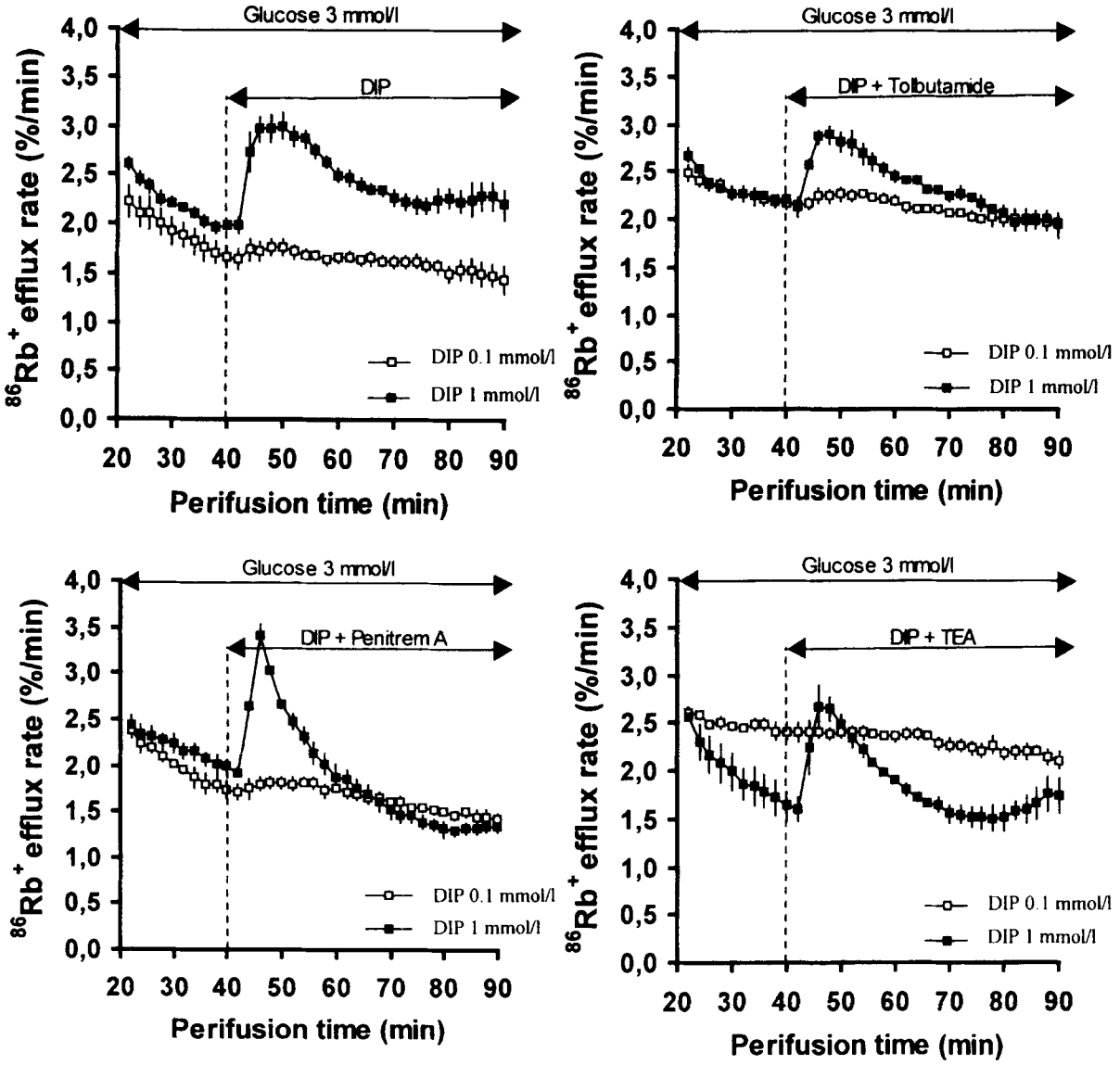

FIGURE 4 Effect of the thiol oxidant diazene dicarboxylic acid *bis* (N-methylpiperazide) (DIP) on $^{86}Rb^+$ efflux rate from islets in the presence of various K^+channel blockers. Tolbutamide (200 µmol/l), Penitrem A (100 nmol/l) and Tetraethylammonium (TEA, 2 mmol/l) were added (40–90 min) in the presence of 3 mmol/l glucose and calcium-free medium. Mean values ± SEM, n = 3

Effects of DIP and DIP^{2+} on the ATP/ADP ratio of pancreatic islets

The effect of DIP, DIP^{2+} and BuOOH on the ATP/ADP ratio of pancreatic islets is shown in Fig. 6. Whereas 0.1 mmol/l DIP produced a small decrease after an incubation time of 30 min, 1 mmol/l DIP caused a decline of about 25 % without reaching significance. Treatment of cells with 1 mmol/l DIP^{2+} and 1 mmol/l BuOOH has no effect on islet ATP/ADP ratio.

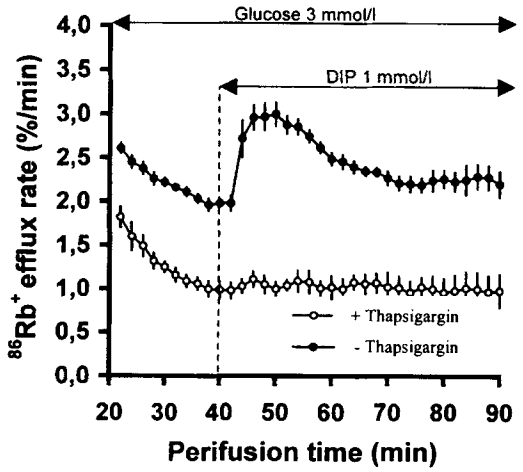

FIGURE 5 Effect of the thiol oxidant diazene dicarboxylic acid *bis*(N-methylpiperazide) (DIP) on $^{86}Rb^+$ efflux rate from islets in calcium-free medium with or without additional preincubation (30 min) with thapsigargin 2 μmol/l. DIP (1 mmol/l) was added from 40–90 min. Glucose concentration was 3 mmol/l throughout the experiment. Mean values ± SEM, n = 3

DISCUSSION

During oxidative stress GSH plays a key role of cellular protection[11]. To provide further understanding of how oxidative stress affects islets function, the effects of DIP and BuOOH, tools being used to lower GSH/GSSG ratio in islets, were studied. Thiol oxidants like DIP have been shown to oxidise intracellular GSH without affecting NAD(P)H, changing the GSH/GSSG status of the cells and lead to a variety of alterations in biological behaviour. Protein thiols may react with diazene oxidizing agents like DIP less rapidly than with GSH[8].

The data of this study indicate that oxidative stress caused by DIP and BuOOH leads to an increased K^+ channel current. Based upon our observations that DIP^{2+}, which does not penetrate into islet cells[12], did not change the K^+ efflux rate it appears that the target for oxidative stress is localized to the inner side of the cell membrane. Opening of ATP-sensitive K^+ channels has emerged as a cardioprotective mechanism[5,13] and intervene to reduce or delay cell death[14]. ATP sensitive channels are found in a number of different tissues where they play distinct physiological functions, but in all these tissues they are blocked by antidiabetic sulfonylureas[15]. Our results demonstrate that the blockade of K^+ ATP-sensitive channels with tolbutamide did not affect the DIP and BuOOH induced K^+ efflux in islets, suggesting that the increased Rb^+ efflux does not simply result from a failure to close K^+ ATP channels. Therefore, we conclude that oxidative stress has no direct effect on the K^+ ATP channels in islet cells.

The stimulating action of oxidants on Rb^+ efflux was somewhat less in the presence of TEA and Penitrem A, suggesting that the TEA- and Penitrem A-sensitive K^+ channels may be involved in the increased potassium efflux. The contribution of these channels to increased $^{86}Rb^+$ efflux appears, however, to be only small. It is possible that to a major part oxidative stress may activate K^+ channels indirectly via liberation of Ca^{2+} from intracellular stores. To test this hypothesis we preincubated islets with thapsigargin, an inhibitor of Ca^{2+} mobilization from Ins (1,4,5)P3-sensitive pools through inhibition of ER Ca^{2+} APTase[16]. Following thapsigargin treatment, elevated Rb^+ efflux was markedly decreased. This indicated to us that inhibition of calcium depletion from intracellular stores prevents the increased K^+ efflux in pancreatic islets during oxidative stress and that in pancreatic islets oxidative stress-mediated potassium efflux is mainly the consequence of intracellular calcium release.

According to observations reported by Maeda et al.[17] low concentrations of ATP enhance IP3 receptor channel activity and Ins(1,4,5)-induced Ca^{2+} release from intracellular stores. Therefore, we measured the ATP/ADP ratio in pancreatic islets treated with the oxidants. These findings, however, indicate that the early depletion of Ca^{2+} from intracellular stores and the release of

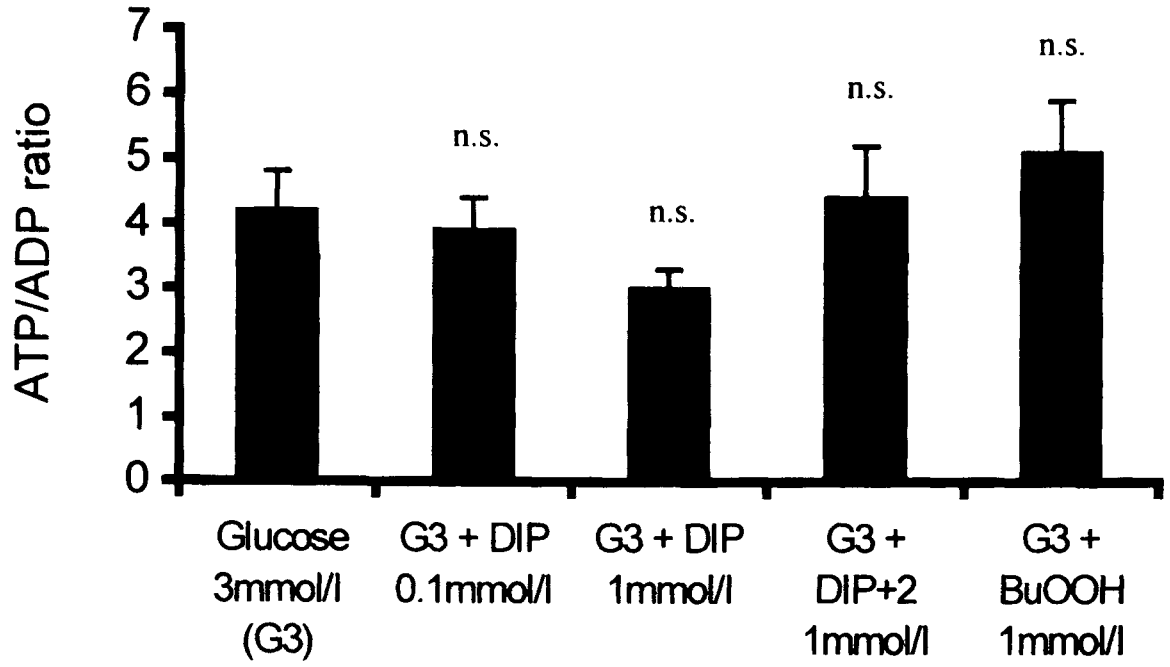

FIGURE 6 Effect of the thiol oxidant diazene dicarboxylic acid bis (N-methylpiperazide) (DIP), -bis N-methyliodide (DIP^{2+}) and BuOOH on the ATP/ADP ratio from rat pancreatic islets. Twenty islets were incubated for 30 min with or without DIP (0.1 mmol/l or 1 mmol/l), DIP^{2+} (1 mmol/l) and BuOOH (1 mmol/l) respectively in KRH buffer containing calcium. Glucose concentration was 3 mmol/l throughout the experiment. Mean values ± SEM, n = 6. ANOVA, Newman-Keuls-test (n.s.: not significant)

K^+ in itself is a phenomenon that is independent of the ATP/ADP ratio or cell damage under the conditions of this study.

In conclusion, multiple mechanisms contribute to cell injury after hypoxia, ischemia/reperfusion and toxic chemicals, but a common final pathway leading to cellular damage may be Ca^{2+} overload after failure of Ca^{2+} stores. One possible mechanism in order to protect the cells is an activation of K^+ channels. In the pancreatic islet such an effect seems to be due to depletion of intracellular calcium stores.

Acknowledgements

The authors express their appreciation to Mrs. I. Hagenloh for skillfull technical assistance.

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