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# Sulfhydryl oxidation induces rapid and reversible closure of the ATP-regulated $K^+$ channel in the pancreatic $\beta$-cell

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Effects of sulfhydryl modification on the ATP regulated $K^+$ channel ($K_{ATP}$ channel) in the pancreatic $\beta$-cell were studied, using the patch clamp technique. Application of the sulfhydryl oxidizing agents thimerosal and 2,2′-dithio-bis(5-nitropyridine) (DTBNP), in micromolar concentrations, caused complete inhibition of the $K_{ATP}$ channel, in inside-out patches. The inhibition was rapid and was reversed by the disulfide reducing agents dithiothreitol and cysteine. Thimerosal, which is poorly membrane permeable, inhibited channel activity, only when applied to the intracellular face of the plasma membrane. In contrast, DTBNP, which is highly lipophilic, caused closure of the $K_{ATP}$ channel and consequent depolarization of the membrane potential, also when applied extracellularly. Our results indicate the presence of accessible free SH groups on the cytoplasmic side of the $K_{ATP}$ channel in the pancreatic $\beta$-cell. These SH groups are essential for channel function and it is possible that thiol-dependent redox mechanisms can modulate $K_{ATP}$ channel activity.

ATP-regulated $K^+$ channel; Sulfhydryl reagent; Thimerosal; Pancreatic $\beta$-cell

## 1. INTRODUCTION

$K^+$ channels characterized by their sensitivity to intracellular ATP ($K_{ATP}$), play an important role in the regulation of insulin secretion from the pancreatic $\beta$-cell [1–3]. Under resting conditions, at glucose concentrations less than 5 mM, the $K_{ATP}$ conductance dominates and therefore determines the membrane potential of the $\beta$-cell [4]. A key event in the glucose stimulation of insulin secretion is the closure of this channel. Closure of the $K_{ATP}$ channel results in depolarization of the cell, $Ca^{2+}$-influx through the voltage-gated $Ca^{2+}$ channel, increase in the cytoplasmic free $Ca^{2+}$ concentration and insulin secretion [3,5]. The $K_{ATP}$ channel is also the target for sulfonylureas, a class of drugs which inhibits channel activity, and are used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM) [6]. The precise signals that generate from glucose metabolism and control the activity of the $K_{ATP}$ channel are still unknown. Currently, a change in the intracellular concentration of ATP or ATP/ADP ratio is believed to be the most important link between fuel metabolism and depolarization of the cell [2,7]. However the regulation of the channel appears to be more complex than that and may involve modulation by protein kinase C, G proteins and changes in the redox potential of the cell. [8–13]. At present little is known about the structure of the $K_{ATP}$ channel protein, as well as about the molecular basis of its regulation.

Many biologically active proteins contain critical cysteine residues. The function of these proteins often depends on the oxidation state of sulfhydryl (thiol) groups (SH groups) [14]. Some proteins are active only when their specific SH groups remain in the reduced form, whereas for the activity of others the disulfide redox state is essential [15,16]. Selective modification of SH groups, has been extensively used to ascertain the relationship between structure and function of many biomolecules. Different types of ion channel proteins also contain SH groups, modification of which may affect channel activity [17,18]. The sulfhydryl reagent thimerosal and some 'reactive disulfides' open intracellular $Ca^{2+}$ channels by oxidizing critical SH groups [18,19]. There is evidence to suggest, that the $K_{ATP}$ channel of mouse skeletal muscle contains functionally important SH groups [20]. The role of SH groups in regulating the activity of the $K_{ATP}$ channel in the pancreatic $\beta$-cell is unknown, although it is known since long that many sulfhydryl reagents stimulate insulin secretion [21–24]. In the present study we demonstrate that the sulfhydryl oxidizing agents, thimerosal and 2,2′-dithio-bis(5-nitropyridine) (DTBNP) induce rapid and reversible closure of the $K_{ATP}$ channel in the pancreatic $\beta$-cell, indicating that this channel contains SH groups essential for the channel activity.

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## 2. MATERIALS AND METHODS

Thimerosal (mercury-[(*o*-carboxyphenyl)thio]-ethyl sodium), DTBNP and dithiothreitol (DTT) were from Sigma. All other chemicals were of analytical grade and were either from Sigma or Merck. DTBNP was dissolved in dimethyl sulfoxide (DMSO), the final concentration of DMSO being not more than 0.1%.

### 2.1. *Preparation of cells*

Pancreatic islets from adult obese mice (ob/ob) were isolated by collagenase digestion and dispersed into single cells by shaking in a $Ca^{2+}$- and $Mg^{2+}$-deficient medium, as previously described [3]. Cells were plated on petri dishes and cultured for 1–3 days in RPMI 1640 medium, containing 11 mM glucose and supplemented with fetal calf serum (10% v/v), penicillin (100 IU/ml) and streptomycin (100 $\mu$g/ml).

### 2.2. *Electrophysiology*

We used inside-out, outside-out and whole-cell modes of the patch-clamp technique [25]. Pipettes were pulled from borosilicate glass and coated with Sylgard resin (Dow Corning) near the tips, fire-polished and had resistances between 2 and 6 M$\Omega$. Single-channel currents were recorded from inside-out or outside-out membrane patches and channel activity was measured at 0 mV membrane potential. The membrane potential was monitored using the whole-cell technique. Current and voltage were recorded using an Axopatch 200 patch-clamp amplifier (Axon Instruments Inc. Foster City, USA). During experiments the current and voltage signals were stored using a VR-100A digital recorder (Instrutech Corp., USA) and a high-resolution video cassette recorder (JVC, Japan). Channel records are displayed according to the convention with upward deflections denoting outward currents. $K_{ATP}$ channel activity was identified on the basis of the sensitivity to ATP and the unitary amplitude (1.5–2 pA).

In all experiments the extracellular solution contained (in mM): 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, and 5 HEPES (pH 7.4 with NaOH). The 'intracellular-like' solution consisted of (in mM): 125 KCl, 1 $MgCl_2$, 10 EGTA, 30 KOH, and 5 HEPES (pH 7.15 with KOH). Patches were excised into nucleotide-free solution and ATP was first added to test for channel inhibition. ATP was then removed and patches were subsequently exposed to the test substances, as indicated in the figures. Mg-ATP (0.1 mM) was present in the intracellular solution for most of the time to reduce run-down of the $K_{ATP}$ channel [26]. The bath had a volume of 0.4 ml and cells were perifused at a rate of 4 ml/min. All test compounds were added to the perifusion medium. Each experimental condition was tested, with identical results, in 3–6 different patches. All experiments were done at room temperature (22°C).

The current signal was filtered at 100 Hz (−3 dB value) by using an 8-pole Bessel filter (Frequency Devices, Haverhill, USA). Single-channel conductance was measured directly from a digital oscilloscope. Figures were made by plotting segments of the records on a chart recorder, scanning the segments using a HP scanner and incorporating them into Corel Draw graphics software program.

## 3. RESULTS

The inhibitory action of thimerosal on $K_{ATP}$ channel activity was rapid and almost complete with 10 $\mu$M of the compound (Fig. 1). The blocking effect was dose-dependent, with a threshold concentration of thimerosal of about 1–2 $\mu$M. At higher concentrations (up to 100 $\mu$M), thimerosal invariably caused complete inhibition of channel activity (data not shown). Replacement of the thimerosal-containing solution with thimerosal-free solution did not cause spontaneous return of channel activity, even when observed for a prolonged period of time (up to 5 min). However, addition of 2 mM DTT, a disulfide reducing agent [27] readily reversed the inhibitory action of thimerosal and caused a substantial return of channel activity. Similar reversal of the inhibitory action of thimerosal was obtained by the disulfide reducing agent cysteine (100 $\mu$M) (data not shown). Although these reducing agents readily reversed the inhibitory effect of thimerosal, they themselves, did not have any effect on channel activity (Fig. 2A). DTT did not block the inhibitory effect of ATP on the $K_{ATP}$ channel (Fig. 2B). We did not observe any effect of thimerosal (10–20 $\mu$M) on the $Ca^{2+}$-sensitive $K^+$ channel (BK channel).

Fig. 3 shows the inhibitory effect of a representative reactive disulfide, DTBNP on the $K_{ATP}$ channel. On addition of DTBNP (20 $\mu$M), there was a rapid and pronounced reduction in channel activity. In control experiments, 0.1% DMSO (solvent for DTBNP) had no effect on channel activity. The inhibitory effect of DTBNP was also partially reversed by the reducing agents DTT (Fig. 3) and cysteine (data not shown).

Since thimerosal is hydrophilic and therefore poorly membrane permeable, it is likely that was affecting SH groups on the cytoplasmic side of the cell membrane. This was confirmed in experiments using the outside-out patch configuration, where the extracellular face of

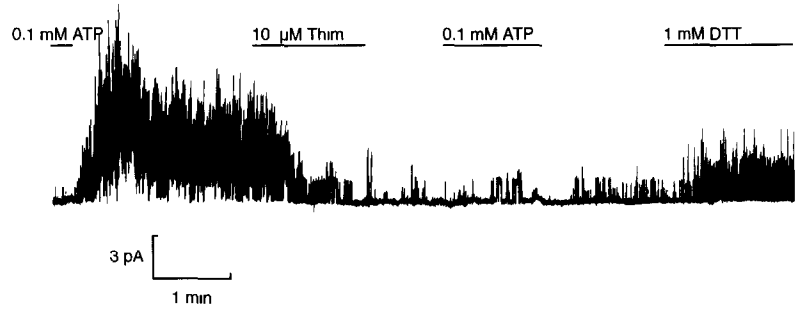


Fig. 1. Effects of thimerosal and DTT on $K_{ATP}$ channel activity. Single-channel recordings from excised inside-out membrane patches obtained from cultured mouse $\beta$-cells. The pipette contained extracellular solution and the cell was perifused with intracellular-like solution. Thimerosal (10 $\mu$M) almost completely blocked the current through the $K_{ATP}$ channel. The blocking effect of thimerosal was not spontaneously reversed upon withdrawal of the substance. Addition of DTT (2 mM) to the same patch, reversed the inhibitory effect of thimerosal.

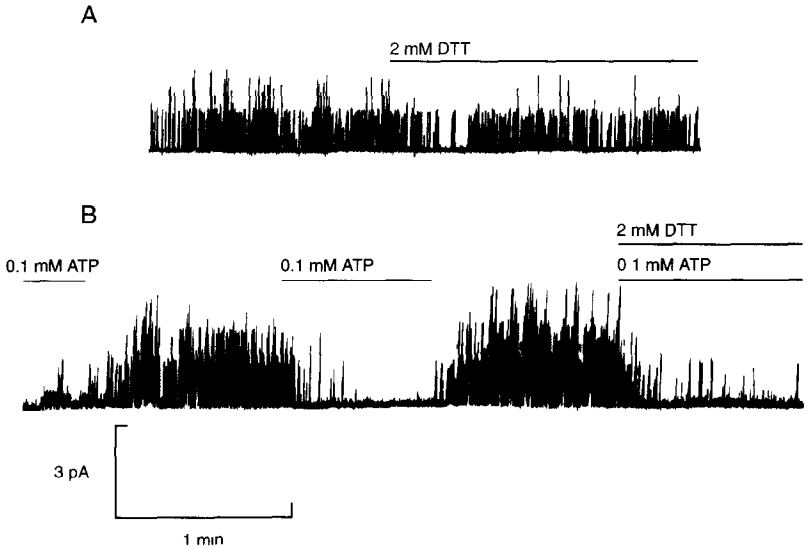


Fig. 2. Single-channel recordings from excised inside-out membrane patches. The the conditions of experiments were as described in the legend to Fig. 1. (A) DTT did not affect $K_{ATP}$ channel activity in the absence of ATP. (B) DTT had no effect on ATP-induced (0.1 mM) inhibition of $K_{ATP}$ channel.

the cell membrane is exposed to the perifusion medium. Addition of a high concentration of thimerosal (100 $\mu$M), no longer blocked the activity of the $K_{ATP}$ channel (Fig. 4A). On the contrary, application of DTBNP, which is highly lipophilic, under the same conditions caused rapid inhibition of the channel (Fig. 4B).

Membrane potentials were recorded from the $\beta$-cell in the whole-cell patch clamp configuration, with the pipette containing ATP-free intracellular-like solution. The decrease in intracellular ATP caused opening of the $K_{ATP}$ channel and membrane repolarization. Thimerosal (up to 100 $\mu$M), applied to the extracellular face of the plasma membrane did not depolarize the cell, whereas subsequent application of DTBNP (50 $\mu$M) did (Fig. 5). Application of DTBNP (50 $\mu$M) alone, without prior addition of thimerosal, had a similar effect on membrane potential (data not shown).

## 4. DISCUSSION

We demonstrate effects of sulfhydryl oxidation, by thimerosal and DTBNP, on $K_{ATP}$ channel activity in the pancreatic $\beta$-cell. Thimerosal inibited channel activity in a dose-dependent manner and was effective in low micromolar concentrations, with maximal inhibitory effect obtained at 10–20 $\mu$M. Thus, thimerosal was a more potent inhibitor of this channel than for example tolbutamide, an example of the first generation of antidiabetic sulfonylureas [6]. The effect of thimerosal was reversed by addition of excess of the disulfide reducing agent DTT or cysteine. This indicates that inhibition of channel activity was caused by sulfhydryl oxidation and not due to a nonspecific effect on the channel. However, the biochemical reaction of thimerosal is not known to be specific for any particular type of SH group. Such

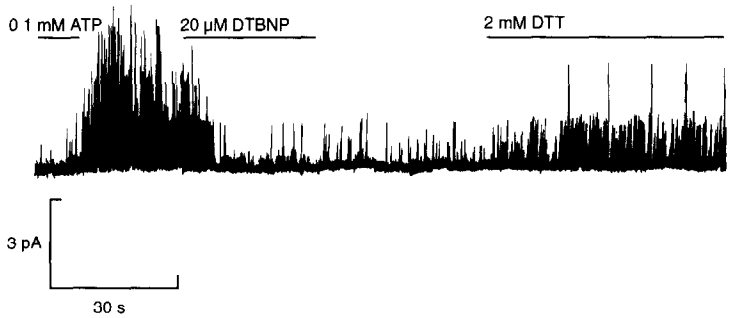


Fig. 3. Effect of DTBNP on $K_{ATP}$ channel activity in a single-channel recording from an excised inside-out patch. The pipette contained extracellular solution and the bath was perfused with intracellular-like solution. Addition of DTBNP (20 $\mu$M) almost totally blocked channel activity. The blocking effect was not reversed following wash out of DTBNP. Upon addition of DTT (2 mM), $K_{ATP}$ channel activity was partially restored

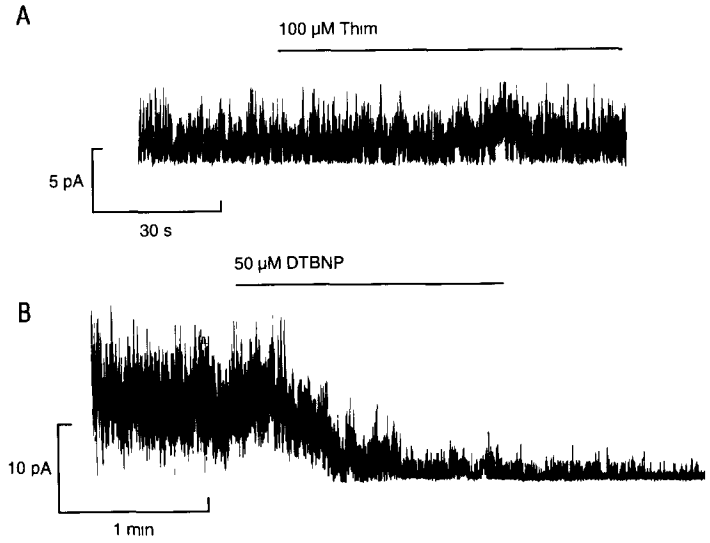


Fig. 4. Single channel recordings from excised outside-out membrane patches. The pipette was filled with intracellular-like solution and the patch was perfused with extracellular medium. (A) Thimerosal (100 $\mu$M) did not affect $K_{ATP}$ currents when applied to the extracellular face of the membrane. (B) Application of 50 $\mu$M DTBNP completely blocked $K_{ATP}$ channel activity in the outside-out membrane patch.

mercurial compounds may also bind to functional groups other than SH groups [28]. For this reason, we tested the effect of another sulfhydryl oxidizing agent, DTBNP. This substance belongs to a class of compounds known as 'reactive disulfides' [29]. DTBNP and related dithiopyridines are almost absolutely specific for free SH groups, which they oxidize through a thiol-disulfide exchange reaction [29,30]. Like thimerosal, DTBNP was also a potent inhibitor of the $K_{ATP}$ channel. In contrast to the effect of thimerosal, inhibition of $K_{ATP}$ channel activity by DTBNP was not reversed to the same extent by the reducing agents. This is not surprising, since it is known that the reaction of DTBNP with SH groups can be irreversible [31].

At the concentrations used in this study, thimerosal was effective only when applied to the cytoplasmic face of the membrane. There was no inhibition of $K_{ATP}$ channel activity and no depolarization of the membrane, when thimerosal was applied to the extracellular side of the membrane. This suggests that the site of the critical SH groups, associated with the $K_{ATP}$ channel, is on the cytoplasmic side of the plasma membrane. Thimerosal, being hydrophilic and poorly membrane permeable, cannot access these SH groups when applied to the extracellular side of the membrane. In contrast to thimerosal, DTBNP is lipophilic and membrane permeable. As expected, application of DTBNP to the extracellular face of the membrane also caused inhibition of the $K_{ATP}$ channel and consequently, membrane depolarization. Since the effect of thimerosal was observed in the ex-

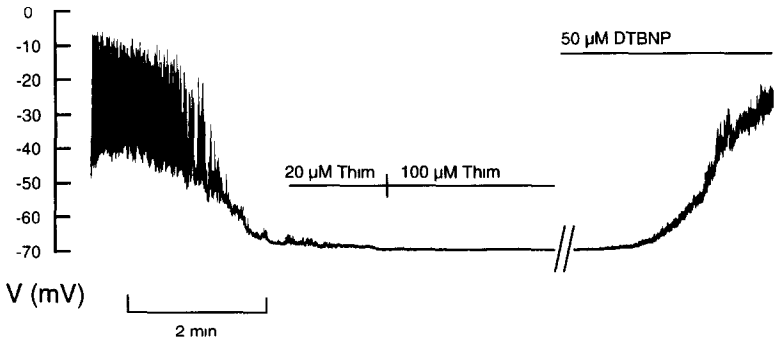


Fig. 5. Whole-cell recordings from $\beta$-cells showing effects of thimerosal and DTBNP on membrane potential. Cells were intracellularly perfused with an ATP-free intracellular-like solution and the bath was perfused with extracellular solution. Prior to the experiments, cells were cultured in media containing 11 mM glucose and because of the short time before the start of an experiment, the cells occasionally exhibited action potentials. As the pipette solution lacked ATP, the cells quickly repolarized to about −70 mV. Thimerosal, in concentrations up to 100 $\mu$M, did not affect membrane potential, whereas DTBNP (50 $\mu$M) readily induced membrane depolarization. Vertical bars in the trace indicate a brake in the record of approximately 3 min.

cised inside-out patch configuration, it is likely to represent a direct interaction with the $K_{ATP}$ channel or a closely associated protein.

These results establish the presence of accessible free SH groups on the $K_{ATP}$ channel in the pancreatic $\beta$-cell, as has been suggested for the $K_{ATP}$ channel in mouse skeletal muscle [20]. Our results indicate that the SH groups are critically important for the regulation of $K_{ATP}$ channel activity. There are many reports of alterations in the function of different ion channels caused by modification of sulfhydryl or disulfide groups on the channel protein. Such effects have been described, for instance, on the nicotinic acetylcholine receptor and on intracellular $Ca^{2+}$ channels such as the ryanodine and the inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) receptor [17–19,30,32]. Noteworthy is, that sulfhydryl oxidation leads to opening of the intracellular $Ca^{2+}$ channels, whereas in the case of the $K_{ATP}$ channel the result of a similar chemical modification is the opposite [19,30].

Closure of the $K_{ATP}$ channel may be the underlying mechanism by which some sulfhydryl reagents, that oxidize relatively superficial SH groups, also strongly stimulate insulin secretion [21–24]. The mechanism of insulin secretion by the antidiabetic sulfonylureas also involves selective inhibition of the $K_{ATP}$ channel. However, the molecular basis of the interaction of the sulfonylureas with the $K_{ATP}$ channel or any associated proteins is still unknown, although it has been suggested that their mechanism of action may involve an interaction with membrane-associated SH groups [33–35]. The present study also suggests that sulfhydryl modification may be a means for developing selective insulinotropic agents, as has been done for the development of some other classes of drugs [36,37]. Whether thiol-dependent redox mechanisms may play a role, also in the physiological regulation of the stimulus-secretion coupling in the pancreatic $\beta$-cell, as has been suggested before [38], is not clear. In principle, however, it is possible that the activity of the $K_{ATP}$ channel may be modulated by metabolism-induced changes in the redox state in the close vicinity of the channel. Such switching mechanisms, by thiol-dependent redox regulation, have been postulated for other biological processes [39–41].

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