J. Membrane Biol. 158, 147–158 (1997)

The Journal of
Membrane
Biology

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Effects of Thiol-Modifying Agents on a K(Ca^{2+}) Channel of Intermediate Conductance in Bovine Aortic Endothelial Cells

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Received: 7 August 1996/Revised: 20 February 1997

Abstract. Ca^{2+}-activated K^+ channels (K(Ca^{2+})) constitute key regulators of the endothelial cell electrophysiological response to $InsP_3$-mobilizing agonists. Inside-out and outside-out patch clamp experiments were thus undertaken to determine if the gating properties of a voltage-insensitive K(Ca^{2+}) channel of intermediate conductance present in bovine aortic endothelial (BAE) cells could be modified by specific sulfhydryl (SH) oxidative and/or reducing reagents. The results obtained first indicate that cytosolic application of hydrophilic oxidative reagents such as 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (0.2 to 5 mM) or [(O-carboxyphenyl)thio]ethyl mercury sodium salt (thimerosal) (0.5 to 5 mM) reduces gradually the K(Ca^{2+}) channel activity with no modification of the channel unitary conductance. The inhibitory action of DTNB (1 to 5 mM) or thimerosal (1 to 5 mM) was not reserved following withdrawal of the oxidative agents, but channel activity could partly be restored by the addition of the SH group reducing agents dithiothreitol (DTT) (5 mM) or reduced glutathione (GSH) (5 mM) in 53% and 50% of the inside-out experiments performed with DTNB and thimerosal respectively. Similar results were obtained using H_2O_2 at concentrations ranging from 500 μM to 10 mM as oxidative reagent. In contrast, the lipid soluble oxidative agent 4,4′-dithiodipyridine (4-PDS) (1 mM) appeared in inside-out experiments less potent than DTNB and thimerosal at inhibiting the K(Ca^{2+}) channel activity, suggesting that the critical SH groups involved in channel gating are localized at the inner face of the cell membrane. This conclusion was further substantiated by a series of outside-out patch clamp experiments which showed that DTNB (5 mM) and thimerosal (5 mM) were unable to inhibit the K(Ca^{2+}) channel activity when applied to the external surface of the excised membrane. Finally, no significant changes of the gating properties of the K(Ca^{2+}) channel were observed in inside-out experiments where the SH group reducing agents DTT and GSH were applied immediately following membrane excision. However, the application of either GSH or DTT was found to partly restore channel activity in experiments where the K(Ca^{2+}) channels showed significant rundown.

Key words: K(Ca^{2+}) channel — Sulfhydryl groups — Oxidation/reduction — Endothelial cells — Free radicals

Introduction

The endothelium constitutes a key regulator of vascular tone by secreting both vasoconstrictors such as endothelin (ED1 among others), and vasorelaxing agents such as the prostacyclin PGI_2 and the major endothelium-derived relaxing factor NO [11]. The production of NO in endothelial cells is known to be stimulated in response to an increase in cytosolic Ca^{2+} [1, 3, 17, 27]. The action of Ca^{2+} involves in this case the activation of a specific enzyme, the NO synthase, which in turn metabolizes L-arginine to citrulline and NO [29]. In addition, there is currently strong evidence that the intracellular Ca^{2+} increase in endothelial cells caused by Ca^{2+} mobilizing agonists such as BK, ATP and histamine, is a biphasic process reflecting, in part, an inositol 1,4,5-trisphosphate ($InsP_3$) mediated release of Ca^{2+} from intracellular stores coupled to a capacitative Ca^{2+} entry from the external medium [14, 30, 37].

Several factors have been reported to modulate the agonist-evoked Ca^{2+} influx in a large variety of nonexcitable cells [20, 33]. In endothelial cells, there are clear

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indications that the agonist-stimulated Ca^{2+} influx is affected by membrane potential, with a hyperpolarization of the cell membrane leading to augmentation and/or stabilization of the Ca^{2+} entry [20, 26, 28, 38]. It has been suggested that this augmentation arises from the increased driving force acting on Ca^{2+} ions under these conditions [6, 7, 28, 33, 36, 38]. This contrasts with the results obtained using excitable cells where Ca^{2+} influx is normally impaired at hyperpolarizing potential values. It has also been demonstrated that the mechanism responsible for Ca^{2+} signalling in endothelial cells is affected by the cell redox state. A recent study by Wesson and Elliot [44] has indicated that short treatments (less than 2 hr) of calf pulmonary endothelial cells with xanthine/xanthine oxidase (a H_2O_2 and O_2^--generating system) or BAE cells with t-buOOH (reactive oxygen species donor) initially cause an inhibition of the agonist-stimulated Ca^{2+} influx, followed over longer time periods by a decrease of the Ca^{2+} release from internal stores and an increase of the basal intracellular Ca^{2+} level [*see also* 39]. This latter effect was attributed to a dysfunction of the plasma membrane Ca^{2+} pump in the presence of oxidative agents. Changes in the cellular redox state, such as the one generated during ischemia and reperfusion, are generally accompanied by a decrease of the GSH and a related increase of the GSSG level. These changes are known to create intracellular conditions where SH groups are likely to be modified. There are already indications that the oxidation state of SH groups is a determining factor in controlling the activity of several ion channels. For instance, the intracellular redox potential of rabbit pulmonary and ear arterial smooth muscle cells was found to modulate the gating of two Ca^{2+}-activated K^+ channels of large conductance (Maxi K(Ca^{2+})) [34], leading to an increase in channel activity in both cell preparations.

Ion channels, especially K^+ selective channels, play a pivotal role in the physiological response of vascular endothelial cells to a large variety of physical and hormonal stimuli. Several studies have shown that the Ca^{2+} response of vascular endothelial cells to vasodilating agents such as BK, ATP or acetylcholine (ACh), involves the development of a K^+ current mediated in BAE cells by a voltage-insensitive Ca^{2+}-activated K^+ channel (K(Ca^{2+})) of intermediate conductance (40 pS) [6, 7, 36]. In fact, K(Ca^{2+}) channels are now recognized as important feedback regulators of the Ca^{2+} influx process in endothelial cells, with K(Ca^{2+}) channel activation leading to hyperpolarization of the cell potential and an enhanced Ca^{2+} entry. Because there is increasing evidence that Maxi K(Ca^{2+}) channels are affected by oxidation of SH groups, we hypothesized that the effects of oxidative agents observed on the Ca^{2+} response of BAE cells may in part involve modifications of the SH groups of the K(Ca^{2+}) channels of intermediate conductance present in these cells. As such, an inhibition of these K(Ca^{2+}) channels would cause a depolarization of the cell potential and result in a decreased Ca^{2+} influx. A study was therefore undertaken to investigate the effect of oxidizing and reducing SH agents on the activity of the voltage-independent K(Ca^{2+}) channel of intermediate conductance found in BAE cells. Our results indicate that the oxidation of cytosolic SH groups by specific reagents leads to an inhibition of the K(Ca^{2+}) channel activity with no effect on the channel unitary conductance.

Materials and Methods

Cell Culture

The details of the BAE cell culture procedure and characterization have been described elsewhere [37]. The cells were tested with endothelial cell-labelling reagents or factor VIII antibodies (Daco, Santa Barbara, CA) and responded positively [19]. BAE cells were grown in Dulbecco's modified Eagle Medium (Gibco) supplemented with 10% newborn calf serum, 3.7 g/l $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin in humidified air with 5% CO_2 atmosphere at 37°C. Cells from serial passage 21–26 were reseeded on microscope cover slips to accommodate the superfusion chamber used for patch measurements.

Patch Clamp Experiments

Single-channel recordings were carried out either in the inside-out or outside-out patch clamp configuration with a List EPC-7 amplifier. The 200 mM KCl patch electrode and cytoplasmic-like solutions had the standard composition as follows (in mM): 200 KCl, 0.5 $MgCl_2$ buffered at pH 7.4 with 25.0 Hepes and 10.0 KOH, for a free Ca^{2+} concentration of approximately 3 μM (estimation based on fura-2 measurements). Ca^{2+}-free bath solutions were prepared by adding 5 mM EGTA to our standard 200 mM KCl solution. Solutions in which the free Ca^{2+} concentration was buffered at 0.2 μM or 0.7 μM were obtained by adding 1 mM EGTA plus 0.655 mM $CaCl_2$ or 1 mM EGTA plus 0.87 mM $CaCl_2$ to standard 200 mM KCl solutions respectively. Patch pipettes were pulled from Pyrex capillaries (Corning 7040) using a David Kopf programmable pipette puller (Model 750) and used uncoated. The resistance of the patch electrode ranged from 4 to 10 MΩ. Current traces were recorded using a VR-10B digital data recorder (Instrutech, Great Neck). For off-line analysis, the signal was sampled at 1.0 kHz and filtered at 500 Hz with two low-pass four-pole Bessel filters (VVS 300B, Frequency Devices, Haverhill, MA) connected in series. Base line drift was corrected through a multiple linear interpolation procedure. When required, the open channel probability, P_o, was estimated from current amplitude histograms on the basis of a binomial distribution as described elsewhere [31]. Time-dependent variations of NP_o, the mean number of open channels with N the maximum number of functional channels in the patch, were calculated from $\langle I \rangle / \Delta I$, with $\langle I \rangle$ the current average value measured over successive time intervals of 5- to 20-sec duration and ΔI the unitary current amplitude. The percentage of inhibition due to channel oxidation, P_i, was estimated from the ratio of the current mean value measured during the first 150-sec exposure to the oxidative agent, relative to the average current value estimated in control conditions. The results were fitted to a standard equation of the form:

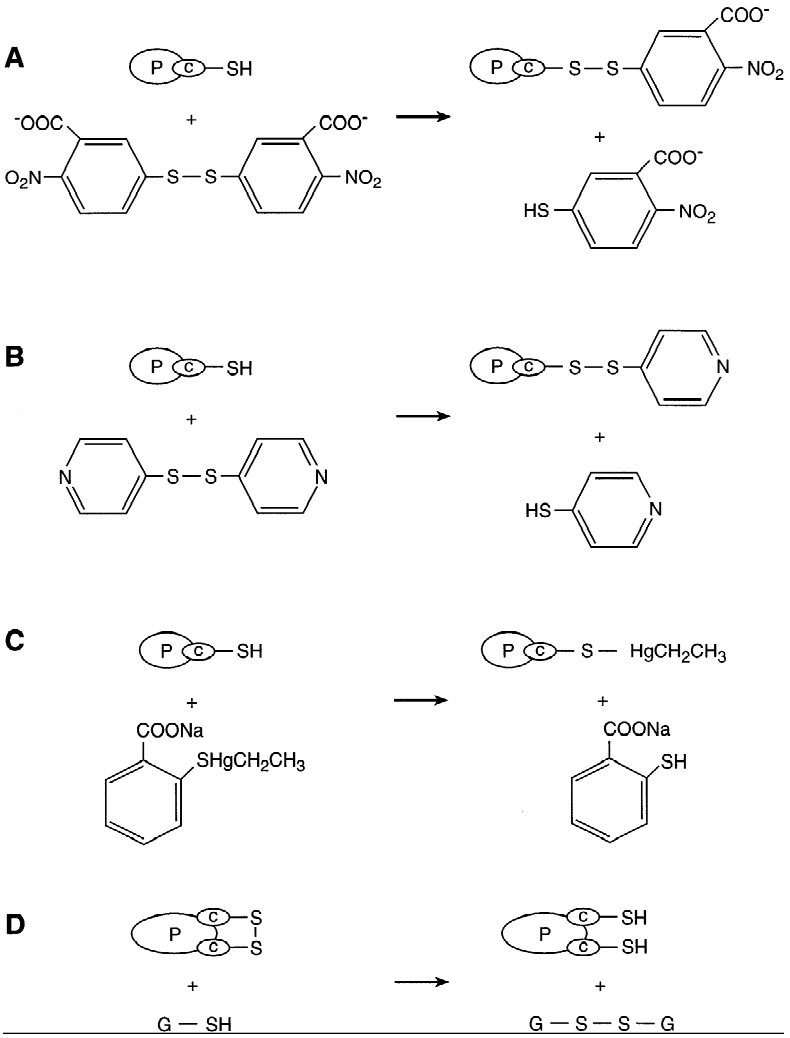

Fig. 1. (*A*) Diagram showing thio-disulphide exchange reaction between the free sulfhydryl groups (SH) on cysteine residues (C) of channel proteins (P) and the SH oxidizing agent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). (*B*) Diagram showing thio-disulphide exchange reaction between SH groups and the lipophilic reagent 4,4′-dithiodipyridine (4-PDS). (*C*) Diagram showing sulfhydryl oxidation by thimerosal ([O-carboxyphenyl) thio]ethylmercury sodium salt), a hydrophilic compound. (*D*) Diagram showing the reduction of disulphide bond (S−S) on channel proteins by the reducing agent reduced glutathione (GSH).

$$P_i = \frac{100}{1 + \left(\frac{X_o}{X}\right)^p} \quad (1)$$

where X is the concentration of the inhibitor, X_o the concentration for half inhibition and P the Hill coefficient. All the experiments were performed at room temperature.

Sulfhydryl Reagents

The reagents were obtained from Sigma (St. Louis, MO). Diagrams of some of the reactions involved in SH group modifications are presented in Fig. 1 [*see* 4]. The oxidation process mediated by DTNB (Fig. 1*A*) and 4-PDS (Fig. 1*B*) is absolutely specific to SH groups in proteins and involves a thiol-disulfide exchange mechanism. DTNB is an acid and, in contrast to 4-PDS, poorly lipid soluble. Thimerosal (Fig. 1*C*) is a mercurial compound that oxidizes SH groups with a high affinity. Like DTNB, thimerosal is a hydrophilic molecule and poorly membrane permeable. Reduction of S−S bonds by molecules such as DTT or GSH is illustrated in Fig. 1*D*. This process leads to the breakdown of intra molecular disulfide and to the formation of free sulfhydryl groups on the channel protein.

Abbreviations

BAE: bovine aortic endothelial cells; BK: bradykinin; DTDP: 2,2′-dithiodipyridine; DTNB: 5,5′-dithio-bis(2-nitrobenzoic acid); DTT: dithiothreitol; EGTA: ethyleneglycol-bis(*b*-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; GSH: reduced glutathione; GSSG: oxidized glutathione; Hepes: N-2-hydroxyethytlpiperazine-N′-2-ethanesulfonic acid; H_2O_2: hydrogen peroxide; NO: nitric oxide; p-CMPS: p-chloromercuri-phenylsulfonic acid; SH: sulfhydryl; Thimerosal: [(O-carboxyphenyl)thio]ethyl mercury sodium salt; 4-PDS: 4,4′-dithiodipyridine; t-buOOH: t-butyl hydroperoxide

Results

Modifications of SH Groups by DTNB and Thimerosal

The involvement of SH groups in the gating properties of the K(Ca^{2+}) channels of intermediate conductance present in BAE cells was first investigated using the SH

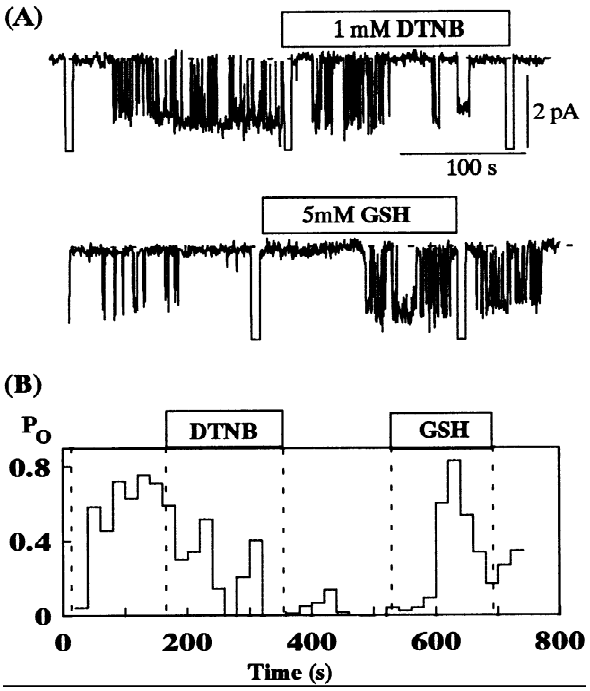

Fig. 2. Effect of DTNB-induced sulfhydryl group oxidation on the activity of the K(Ca^{2+}) channels in BAE cells. (*A*) Example of inside-out recording measured in symmetrical 200 mM KCl conditions. The free Ca^{2+} concentration in the bathing medium was estimated at 3 μM. The sulfhydryl oxidizing agent DTNB (1 mM) caused an irreversible inhibition of channel activity. Channel activity could be recovered by bath application of the reducing agent GSH (5 mM). (*B*) Changes in open channel probability computed from the current record presented in *A*. P_o estimations were calculated from current averages taken over successive 20-sec periods. Pipette potential: +60 mV. Signal filtered at 500 Hz.

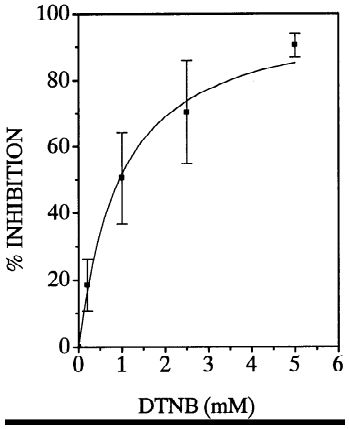

Fig. 3. Percentage of inhibition of the K(Ca^{2+}) channel activity as a function of the DTNB concentration. Continuous line calculated according to equation (1) with $X_o = 1$ mM and $P = 1.0$. Percentage of inhibition computed from the ratio of the average current value taken over the first 150 sec following DTNB exposure, relative to the current mean value measured in control conditions prior to DTNB application ($n = 3$ for 0.2 mM, 2.5 mM and 5 mM; $n = 9$ for 1 mM).

modifying reagent DTNB. An example of the effect of 1 mM DTNB on channel activity is presented in Fig. 2*A*. The related changes in open channel probability averaged over 20-sec periods is illustrated in Fig. 2*B*. Channel inhibition was observed in 19 (95%) of the 21 inside-out experiments performed following DTNB (1 to 5 mM) application for time periods ranging from 20 to 200 sec. There was no detectable change in the channel unitary conductance. Channel activity could not be restored with the withdrawal of DTNB from the cytosolic-like bathing medium, suggesting a high affinity dissociation constant or covalent modifications under these conditions. However, channel activity was partly restored by bath application of the SH reducing agent GSH (5 mM). For instance, in the experiment illustrated in Fig. 2*A*, the open channel probability was equal to 0.74 in control conditions prior to DTNB, to 0.06 after DTNB exposure and to 0.4 following GSH application. These observations indicate that the observed inhibitory effect of DTNB is specifically related to the oxidation of critical SH groups. Not all recordings showed reversibility upon addition of SH reducing agents. In 9 (47%) of the 19 inside-out experiments performed at DTNB concentrations greater than 1 mM, exposure for time periods ranging from 20 to 200 sec resulted in an irreversible, GSH-insensitive loss of channel activity. However, recovery of channel activity following DTNB withdrawal was observed in 3/3 of the experiments carried out at 0.2 mM DTNB. The dose-response curve to DTNB for channels which showed GSH-reversibility is presented in Fig. 3. The results could be fitted to Eq. (1), with $X_o = 1.0$ mM and $p = 1.0$, for half inhibition occurring at 1 mM. The channel specificity of the DTNB inhibitory effect was also apparent in experiments where both the voltage insensitive K(Ca^{2+}) channel and the Ca^{2+}-independent inward rectifying K^+ channel, IK_1, already identified in BAE cells, were present [*see* 36]. The single-channel recording in Fig. 4*a* shows a typical Ca^{2+}-insensitive IK_1 type channel activity measured in low Ca^{2+} conditions (0.2 μM). Inward rectification was confirmed by the absence of detectable outward currents despite the presence of 200 mM KCl + 0.5 mM $MgCl_2$ in the bathing medium (*data not shown*). In Fig. 4*b*, increasing the bath Ca^{2+} concentration to 3 μM resulted in the activation of two K(Ca^{2+}) channels the activity of which was superimposed to the original IK_1 current fluctuations. However, the IK_1 channel activity remains insensitive to DTNB at concentrations sufficient to cause a complete inhibition of the K(Ca^{2+}) channels (Fig. 4*b* compared to Fig. 4*c*). These results confirm that the action of DTNB does not involve a nonspecific interaction with SH groups on the cell membrane. Inhibition of the K(Ca^{2+}) channel activity due to SH group oxidation was also observed in inside-out experiments in the presence of the hydrophilic

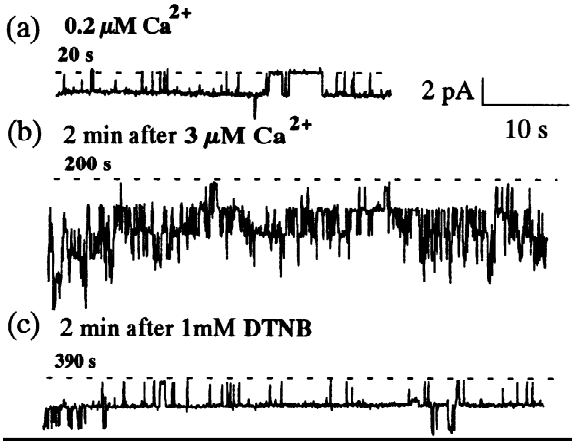

Fig. 4. Channel specificity of the DTNB action. (*a*): Inside-out recording of an IK_1 type Ca^{2+}-insensitive inward rectifying K^+ channel. This segment was taken 20 sec after the beginning of the experiment (*b*): Additional single-channel activity resulting from the activation of two K(Ca^{2+}) channels in 3 μM free Ca^{2+} conditions. Segment taken 200 sec after the beginning of the experiment (*c*): Exposure to DTNB (1 mM) caused a specific inhibition of the K(Ca^{2+}) channels, while leaving the IK_1 channel activity unchanged. Segment taken 390 sec after the beginning of the experiments. Signal filtered at 500 Hz. The pipette potential was +60 mV throughout. Dotted lines refer to the zero current level.

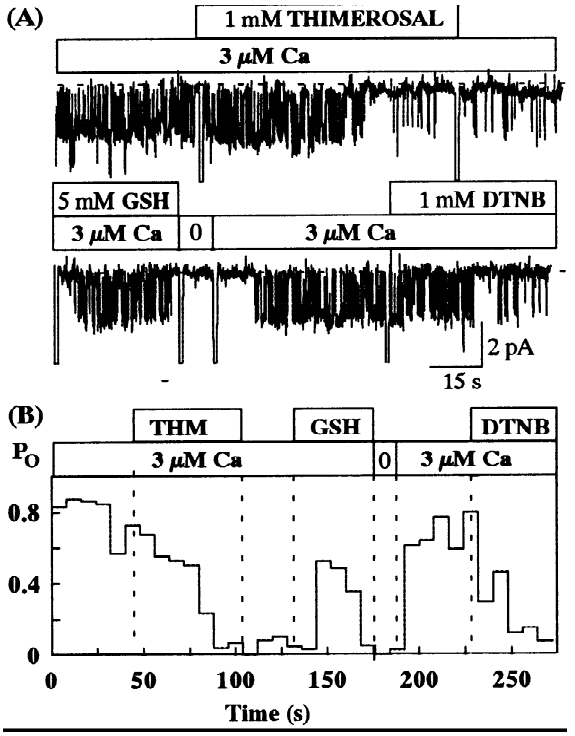

Fig. 5. Effect of thimerosal-induced sulfhydryl group oxidation on the activity of the K(Ca^{2+}) channels in BAE cells. (*A*) Example of inside-out single channel recording measured in symmetrical 200 mM KCl conditions. The free Ca^{2+} concentration in control bath conditions was 3 μm. The sulfhydryl oxidizing agent thimerosal (THM, 1 mM) caused an irreversible inhibition of channel activity. Channel activity could be recovered by bath application of the reducing agent GSH (5 mM). The channel activated by GSH was Ca^{2+}-dependent, since bath perfusion with a Ca^{2+} free solution (0) resulted in the absence of channel opening. Finally, DTNB at 1 mM appeared as potent as thimerosal in inhibiting channel activity. (*B*) Changes in open channel probability computed from the current record presented in *A*. NP_o estimations were calculated from current averages taken over successive 8-sec periods. Signal filtered at 500 Hz. Voltage in patch electrode was maintained at +60 mV throughout. In (*A*), the zero current level corresponds to dotted lines.

reagent thimerosal (500 μM to 5 mM). Figure 5*A* illustrates an example of single-channel recording where thimerosal (1 mM) caused a decrease of the open channel probability, P_o, from 0.8 to .06 within 60 sec. Partial channel recovery ($P_o = 0.6$) was initiated in this case by bath application of GSH (5 mM). The Ca^{2+} dependency of the channel activated by GSH could be confirmed by the absence of channel activity following perfusion with a Ca^{2+}-free bath solution. It is clear also from this recording that DTNB is as potent as thimerosal. Similar results were obtained in 7 out of the 14 inside-out experiments performed with thimerosal at concentrations ranging from 1 to 5 mM. However, in 50% (7/14) of experiments performed with thimerosal, GSH failed to mediate a recovery of channel activity. In contrast to DTNB, thimerosal constituted an effective inhibitory agent of the IK_1 channel. For instance, the recording in Fig. 6*A* shows that the addition of thimerosal caused the complete inhibition of the Ca^{2+}-insensitive IK_1 channel. The absence of K(Ca^{2+}) channels in this case was clearly established by the lack of channel activation following an increase of the bath Ca^{2+} concentration from 0.2 to 3 μM. Furthermore, perfusion with a solution containing GSH (5 mM) succeeded to reverse the thimerosal-induced inhibitory effect. These observations point toward a difference in SH group contribution to channel gating between the Ca^{2+}-insensitive IK_1 channel and the voltage independent K(Ca^{2+}) channel of intermediate conductance present in BAE cells.

Modification of SH Groups by 4-PDS and H_2O_2

DTNB and thimerosal constitute examples of poorly lipid soluble SH modifying agents. Complementary experiments were performed where oxidation of SH groups was accomplished using the membrane permeant sulfhydryl reagents 4-PDS and H_2O_2. Figure 7 illustrates a typical inside-out recording in which 4-PDS (1 mM) was used as SH modifying agent. Clearly, 4-PDS appeared less effective than DTNB at decreasing K(Ca^{2+}) channel activity, as channel openings were still observed after a 6.5-min exposure to the oxidative agent (Fig. 7*c*). A similar behavior was observed in 3 out of the 4 inside-out experiments carried out at 1 mM 4-PDS. Furthermore, removal of 4-PDS resulted in a slow recovery of the channel activity (Fig. 7*d*) with the open channel prob-

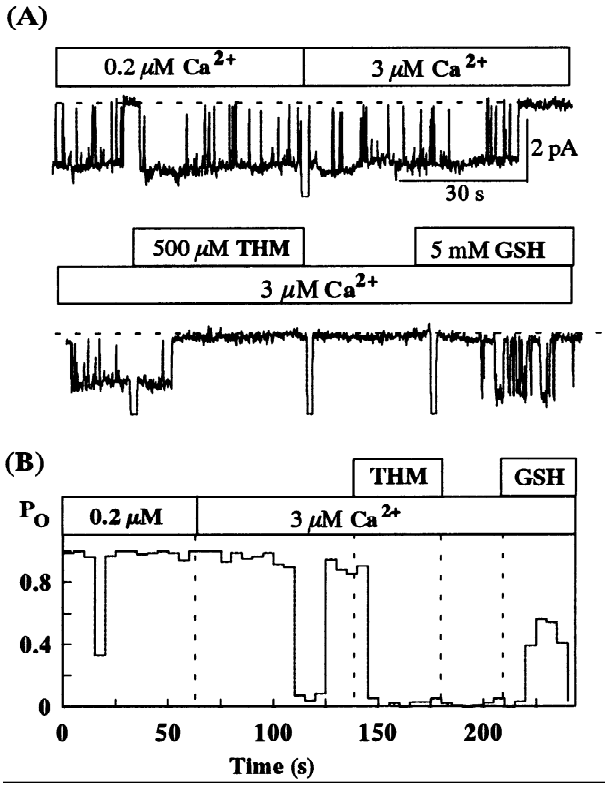

Fig. 6. Effect of thimerosal on the Ca^{2+}-insensitive inward rectifying IK_1 channel in BAE cells. (*A*) Control activity measured in the inside-out configuration at a free cytosolic Ca^{2+} concentration of 0.2 μM. Note the absence of additional channel activity after increasing the Ca^{2+} concentration from 0.2 μM to 3 μM. Thimerosal (THM) at 500 μM caused a complete inhibition of channel activity and this effect was not reversed following thimerosal withdrawal. This effect could be reversed however following GSH application. (*B*) Changes in open channel probability computed from the current record presented in *A*. P_o estimations were calculated from current averages taken over successive 5-sec periods. Signal filtered at 500 Hz. The pipette potential was +60 mV throughout. In (*A*), the zero current level corresponds to dotted lines.

ability increasing from 0.12 to 0.31. Similar increases were found in 6 out of 8 experiments performed with 4-PDS at concentrations of 0.5 and 1 mM. This increase in channel activity could be further enhanced by applying a reducing agent such as GSH (Fig. 7*e*). In contrast, a 1 mM DTNB application caused a complete closure of the same channels within less than a 1 min (Fig. 7*f*). These results provided clear indications for an interaction 4-PDS/SH group that is weaker than that taking place with DTNB or thimerosal. Oxidation by the membrane permeant oxidative agent H_2O_2 was also found to modulate channel activity. For instance, the inside-out recording presented in Fig. 8*A* together with the associated NP_o measurements in Fig. 8*B* provided evidence for a reduction in K(Ca^{2+}) channel activity by H_2O_2. Channel inhibition was recorded in 28 (95%) of the 30 inside-out experiments performed at H_2O_2 concentrations ranging from 0.5 to 10 mM. In two experiments only did H_2O_2 (500 μM and 10 mM, respectively) fail to modify channel gating. Recovery of channel activity resulting from the application of either GSH (1 mM) or DTT (1–5 mM) was measured in 15 (50%) of the 28 experiments where H_2O_2-induced K(Ca^{2+}) channel inhibition was observed. In some cases, (9/28), the effect of H_2O_2 consisted of an initial channel activation followed by a time-dependent inhibition. This increase in single channel activity was characterized by significant changes in the channel gating behavior with the appearance of long open and closed time intervals. It was concluded on the basis of these inside-out experiments that hydrophilic SH modifying agents constitute more potent inhibitors of the K(Ca^{2+}) channels in BAE cells than lipophilic reagents.

Localization of the SH Groups

The localization of the critical SH groups responsible for the inhibitory action of the hydrophilic oxidative agents DTNB and thimerosal was investigated in a series of patch clamp experiments carried out in the outside-out patch excised configuration. Figure 9 illustrates the result of two experiments performed in the inside-out and outside-out patch clamp configuration, respectively, with DTNB (5 mM) as oxidative agent. It is apparent from these recordings, that the application of the oxidative reagent was remarkably less effective at inhibiting channel activity in outside-out than in inside-out experiments. The recording in Fig. 9*A* confirmed the results of Fig. 2*A* while providing evidence for an enhanced rate of inhibition with increasing concentrations of DTNB (5 mM). For instance, total inhibition of the K(Ca^{2+}) channels was obtained within less than 80 ± 36 sec at 2.5 mM ($n = 3$) and 30 ± 10 sec at 5 mM ($n = 3$) DTNB respectively. In contrast, the panel (*b*) in Fig. 9*B* indicates that experiments performed in the outside-out configuration yielded partial inhibition only (25 ± 4%, $n = 5$) despite exposure to 5 mM DTNB for more than 15 min. A similar inhibitory pattern was obtained using thimerosal as oxidative agent (Fig. 10). As in Fig. 9, there was a marked difference in the potency of the SH group modifying agent in inhibiting channel activity between experiments carried out in the outside-out or inside-out configurations. Fig. 10*A* and *B* show that there were no significant changes (8 ± 5%, $n = 5$) in channel gating after a 3-min thimerosal application in outside-out experiments, while the same thimerosal concentration led to an almost instantaneous inhibition of channel activity when used in the inside-out configuration. An absence of thimerosal-induced inhibition was also observed in 4 additional outside-out experiments performed at a thimerosal concentration of 5 mM. These results strongly suggest that SH groups responsible for the oxidation-dependent inactivation of the K(Ca^{2+}) channels in BAE cells are located at the inner face of the cell membrane.

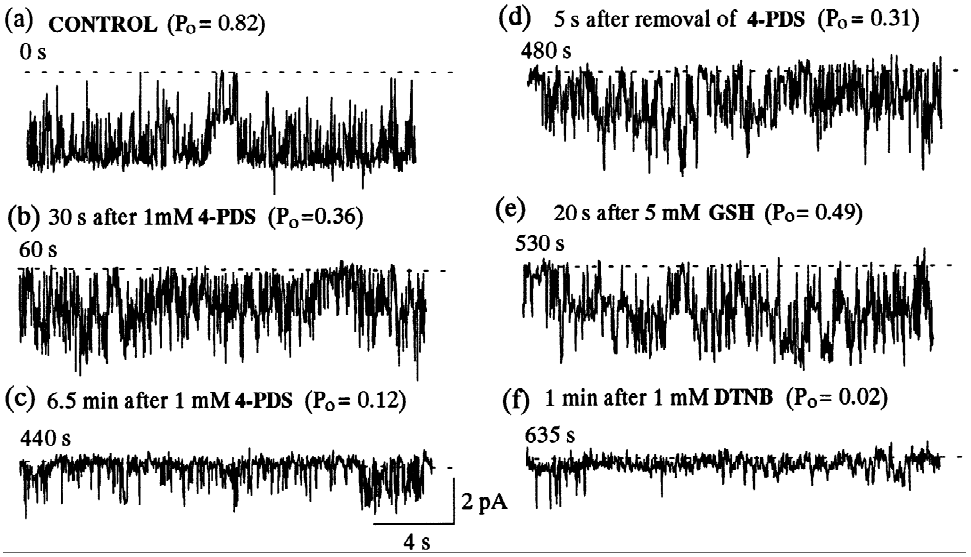

Fig. 7. K(Ca^{2+}) channel oxidation by the lipid soluble SH reagent 4-PDS. Single-channel recording obtained in the inside-out configuration. (*a–c*): Progressive inhibition of channel activity following exposure to 1 mM 4-PDS. The records (*a*), (*b*) and (*c*) were taken 0, 30 and 440 sec after the beginning of the experiment respectively. (*d*): Partial recovery from inhibition following withdrawal of 4-PDS, indicating that the lower activity level after 6.5 min was not due to rundown. Record taken 480 sec after the beginning of the experiment. (*e*): Enhancement of the channel activity in the presence of the reducing agent GSH. Record taken 530 sec after beginning of the experiment. (*f*): Fast inhibitory action when DTNB was used as oxidative agent. Record taken 635 sec after the beginning of the experiment. Pipette potential was 60 mV. Signal filtered at 500 Hz. Dotted lines indicate the zero current level.

Effect of SH Reducing Agents

Single-channel recordings performed either in the inside-out or outside-out configuration have failed to indicate an effect of GSH or DTT on channel gating when applied immediately following membrane excision. However, in cases where the K(Ca^{2+}) channels showed significant rundown in inside-out experiments, application of either GSH or DTT was found to partly restore channel activity (Fig. 11). These observations would support the proposal that channel rundown in these cases is somehow related to an oxidation of the K(Ca^{2+}) channels.

Discussion

The present study shows that the oxidation of SH groups by specific oxidizing agents leads to an inhibition of the K(Ca^{2+}) channels present in BAE cells with no effect on the channel unitary conductance. Inhibition of channel activity by hydrophilic agents was observed after cytosolic but not extracellular applications of the thiol modifying reagents. The effect of DTNB appeared specific to K(Ca^{2+}) channels, with little or no oxidation-related inhibition of the inward rectifying IK_1 channel. In contrast, thimerosal was found to be a potent inhibitory agent of IK_1. Finally, partial recovery from spontaneous K(Ca^{2+}) channel rundown in patch excised experiments was initiated using the SH group reducing agents GSH and DTT.

Sulfhydryl Group Modifications and Channel Activity

The oxidative state of SH groups is known to constitute a determinant factor in ion channel activity. Ruppersberg et al. [35] have shown that replacing a cysteine by a serine in the C terminal region of the $K_v1.4$ channel eliminated the effects of reducing or oxidative agents on the channel inactivation process. The importance of SH groups to channel function was also confirmed for a variety of Ca^{2+}-dependent ion channels. For example, the oxidation of SH groups by H_2O_2 (2–5 mM) has been reported to cause an increased open probability of the Ca^{2+}-activated-Ca^{2+}-releasing channel from the cardiac sarcoplasmic reticulum [2]. There are also indications that oxygen-derived free radicals and SH oxidizing agents stimulate a Ca^{2+}-dependent nonselective (I_{NS}) cation current in guinea pig ventricular myocytes when applied externally [22]. The data obtained for these Ca^{2+}-dependent channels are clearly at variance with the results presented in this work where oxidation of SH groups caused channel closure. Several studies have, however, already reported inhibition of channel activity following oxidation. For instance, the hydrophilic sulfhydryl-oxidizing agent, thimerosal, was found to pro-

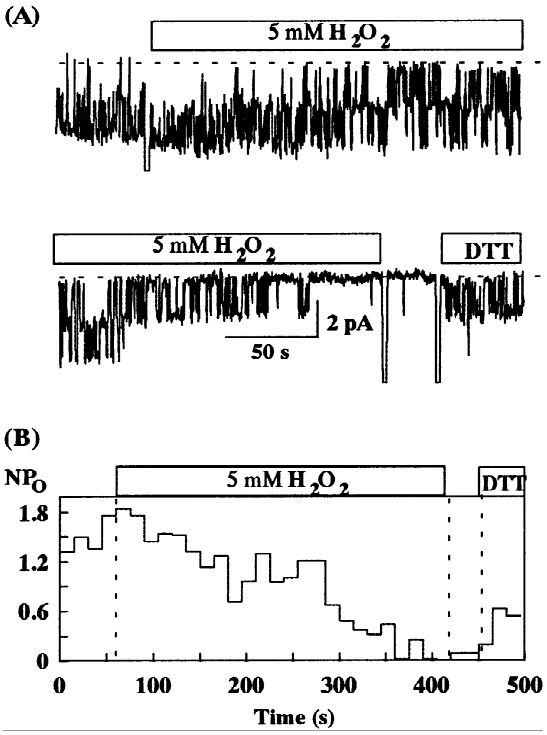

Fig. 8. Effect of H_2O_2 on the activity of the K(Ca^{2+}) channel in BAE cells. (*A*) Inside-out experiments performed in symmetrical 200 mM KCl. The free Ca^{2+} concentration in the bathing medium was estimated at 3 μM throughout. Bath application of 5 mM H_2O_2 produced a progressive decrease in channel activity, which could be partly recovered in the presence of the SH group reducing agent DTT (5 mM) (*B*). Time variation of NP_o computed from the current recording presented in *A*. NP_o was calculated from current averages taken over successive 15-sec periods. Pipette potential was +60 mV. Signal filtered at 500 Hz. Dotted lines marked the zero current level in (*A*).

duce a reversible blockade of a Ca^{2+}-activated nonselective cation channel in brown fat cells [23]. A recent work by Coetzee et al. [5] on K_{ATP} channels, has also provided evidence for an inhibition of channel activity in the presence of internal thiol oxidative agents such as DTNB and pCMPS. Similarly, an inhibition of channel activity by DTDP and by thimerosal was observed on cloned rabbit smooth muscle L-type Ca^{2+} channels [4]. In contrast to the results presented in Figs. 9 and 10, the free sulfhydryl groups in the latter case appeared accessible from the external side. SH group modifying agents have also been shown to differentially modulate the behavior of voltage dependent Ca^{2+}-activated K^+ channels of large conductance (Maxi K(Ca^{2+})). Inside-out experiments have indicated that SH group oxidation by DTNB (1 mM) causes an increase in the activity of Maxi K(Ca^{2+}) channels present in rabbit pulmonary and ear arterial smooth muscle cells [34]. In both cases, the effect of internal oxidative agents could be restored by applying SH reducing agents. In contrast, a recent work by Wang et al. [42] demonstrated that the Maxi K(Ca^{2+}) in equine tracheal myocyte is activated by GSH and inhibited by SH oxidation. As in the present case, it was concluded that the SH groups responsible for this regulation were accessible from the intracellular side. An important inhibition of channel activity was also reported for the Ca^{2+}-activated K^+ channel of intermediate conductance present in erythrocytes following internal application of menadione or menadione analogues [12, 15]. These observations are in agreement with the results reported in this work on the K(Ca^{2+}) channel in BAE cells, where SH group oxidation by DTNB and thimerosal was found to inhibit channel activity. The reasons for the observed diversity in the response of ion channels to SH group modifications remains unclear. In some cases, the action of oxidative agents on ion channels may not be direct. For instance, an indirect activation by reactive oxygen species of voltage-dependent K^+ channels was observed in the human lung adenocarcinoma cell line A549 [24]. It was suggested in this case that the oxidative agents activate a protein kinase which in turn affects the K^+ channels. It remains therefore possible that the effect of SH oxidative agents observed in inside-out experiments on the K(Ca^{2+}) channel in BAE cells resulted from an indirect action of the agents on a protein involved in K(Ca^{2+}) channel regulation. This action would need however to be specific to the K(Ca^{2+}) channel, since no effect of DTNB was observed on the inward rectifying IK_1 channel (*see* Fig. 4). Furthermore the possibility of a cytosolic regulatory protein can be ruled out since the effects of oxidative agents were in this study most evident in cell excised patch clamp experiments.

Nature of the SH Groups

The nature of the SH group modification is a function of the particular physico-chemical properties of the SH oxidizing and/or reducing agents used [10]. Our inside-out experiments show that DTNB (1 mM) and thimerosal constitute potent inhibitory agents of channel activity. Half inhibition with DTNB was observed at 1 mM (Fig. 3), a value in agreement with the results obtained for Maxi K(Ca^{2+}) channels [34], but higher than that reported by Coetzee et al. [5] for the K_{ATP} channel in guinea pig ventricular cells. Our results also showed that the sulfhydryl group modifying agent 4-PDS appeared less effective than both thimerosal and DTNB. In fact, complete inhibition of channel activity was never observed in the presence of 4-PDS at a high concentration (1 mM), regardless of the duration of the application period (5 to 10 min). That the targets of the DTNB and thimerosal were SH groups was confirmed by the ability of GSH and/or DTT to reverse the inhibitory action of DTNB and thimerosal when applied at 1 and 5 mM respectively (Figs. 2, 5, 9 and 10). DTNB and 4-PDS are known to oxidize free sulfhydryl groups through a thiol-

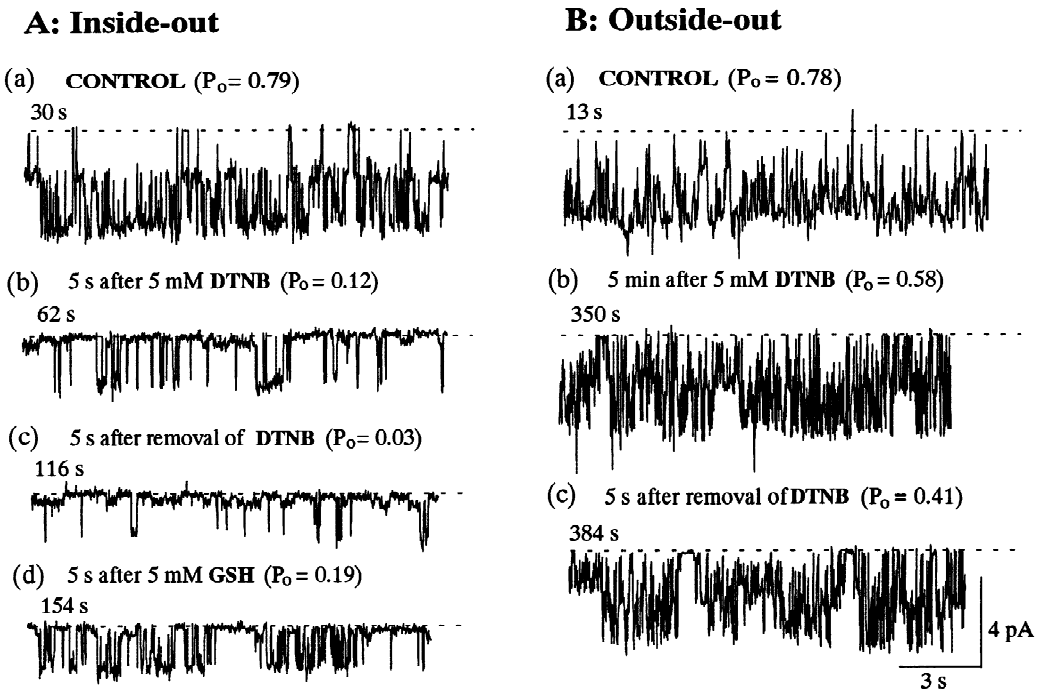

Fig. 9. Localization of the K(Ca^{2+}) channel SH groups affected by the oxidative agent DTNB. (*A*) Example of a marked inhibition of channel activity (*a–d*) observed in inside-out experiments following a brief application of DTNB (5 mM) at the membrane inner face. (*B*) Example of outside-out recording where a prolonged (5 min) bath application of DTNB (5 mM) caused a weak inhibition only of channel activity (*a–c*). Pipette potential of +60 and −60 mV in *A* and *B* respectively. Signal filtered at 500 Hz. Starting time of each current trace relative to the beginning of the experiment is shown on the left. Dotted lines refer to the zero current level.

disulphide exchange mechanism. In contrast, thimerosal binds to SH groups via a S-Hg interaction (Fig. 1). Thus the relative potency of DTNB and thimerosal compared to 4-PDS cannot be explained by the nature of the SH group modifications. However, thimerosal is a highly hydrophilic molecule and as such should have limited access to the hydrophobic domains of the K(Ca^{2+}) channel protein [10, 21]. Similarly, measurements on red cells have confirmed that DTNB is poorly membrane permeant and reacts preferentially with surface SH groups [40]. In contrast, 4-PDS is substantially less water soluble than DTNB despite structural similarities (Fig. 1). These observations suggest that the differences between the inhibitory actions of DTNB, thimerosal and 4-PDS may be linked to their lipid solubilities. As a result, DTNB and 4-PDS are likely to interact with different populations of sulfhydryl groups, 4-PDS reacting more readily with the SH groups localized within the lipid region of the cell membrane.

Numerous studies have established that the SH groups of proteins undergo oxidation with the formation of inter- or intramolecular disulfide bonds by the action of agents such as H_2O_2 [43]. The recordings presented in Fig. 8 provided evidence for an inhibitory action of H_2O_2 on the K(Ca^{2+}) channel activity. However, in 9 out of the 28 experiments performed with H_2O_2, the observed inhibition involved complex fluctuation patterns with an initial increase in the open channel probability followed by a time dependent decrease of the channel activity (*data not shown*). Such complex behavior may be indicative of the contribution coming from distinct SH groups to the overall channel gating process. The oxidation of K(Ca^{2+}) channel by H_2O_2 is likely to yield intramolecular disulfide bond formation. As such, this mechanism differs from the SH group modifications generated by agents like DTNB, thimerosal and 4-PDS (*see* Fig. 1). It is possible therefore that the complexity of the H_2O_2 action arises from the particular nature of the SH group interactions initiated in this case. In addition, because H_2O_2 is freely membrane permeant, the SH groups available to react with H_2O_2 include both hydrophilic and hydrophobic sites. This would suggest that H_2O_2 can interact with SH groups potentially distinct from those affected by DTNB, thimerosal or 4-PDS.

Finally, our results indicate that the reducing agents DTT and GSH do not affect K(Ca^{2+}) channel activity when applied immediately following membrane excision. These observations support the proposal that K(Ca^{2+}) channels are normally maintained in a reduced state, since additional attempts to reduce SH groups by DTT or GSH following patch excision failed to affect channel activity. The possibility that target SH groups

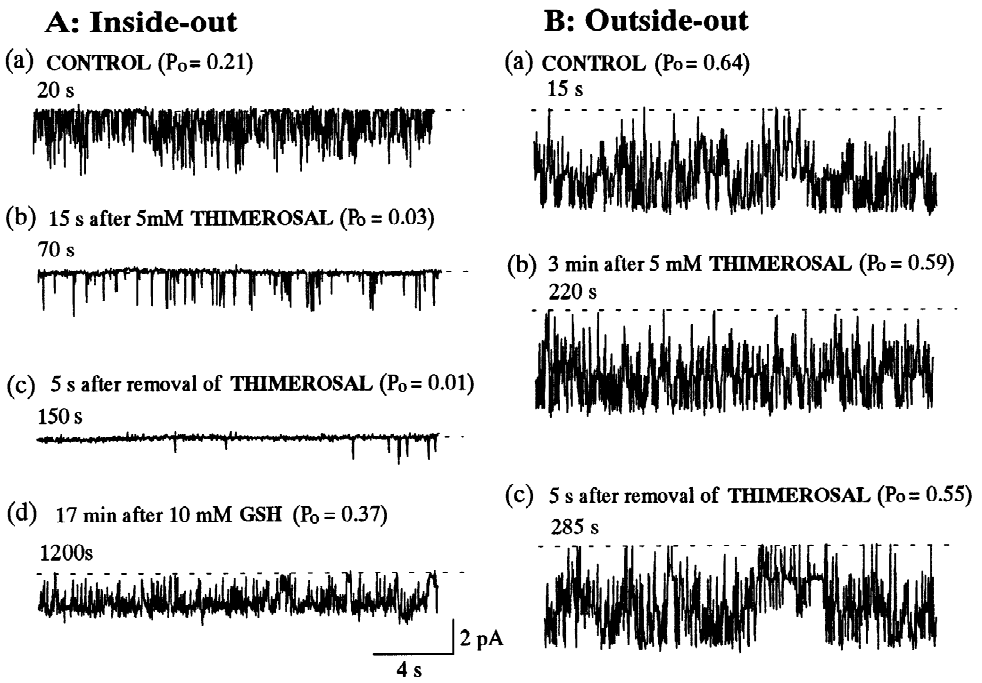

Fig. 10. Localization of the K(Ca^{2+}) channel SH groups using the hydrophilic oxidative agent thimerosal (*A*) 86% inhibition of channel activity (*a–b*) initiated in the inside-out configuration following a 15-sec bath application of thimerosal (5 mM) (*B*) Prolonged (3 min) bath application of thimerosal (5 mM) failed to cause a significant inhibition of channel activity (*a–c*) in the outside-out configuration. Pipette potential of +60 and −60 mV in *A* and *B* respectively. Signal filtered at 500 Hz. Starting time of each current trace relative to the beginning of the experiment is shown on the left. Dotted lines refer to the zero current level.

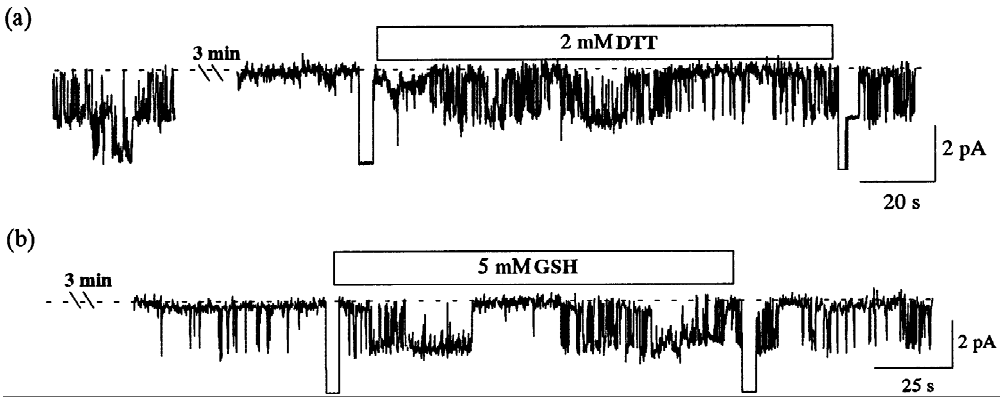

Fig. 11. Recovery from K(Ca^{2+}) channel rundown in the presence of the reducing agents DTT (*a*) or GSH (*b*). Examples of single-channel recordings where patch excision led to a gradual decrease in single channel activity (rundown). However, bath application of the SH group reducing agents DTT or GSH resulted in a partial recovery of the original channel activity. Pipette potential was equal to +60 mV throughout. Signal filtered at 500 Hz.

for GHS or DTT interactions only become accessible after oxidation of the K(Ca^{2+}) channel can not, however, be currently ruled out. Furthermore, in inside-out patch clamp experiments where spontaneous K(Ca^{2+}) channel rundown was observed, the addition of either DTT or GSH succeeded in partly recovering channel activity (*see* Fig. 11). On the basis of these observations it was concluded that the rundown phenomenon taking place in this case resulted in part from the oxidation of critical SH groups localized either on the K(Ca^{2+}) channel or on some regulatory protein. This conclusion is further supported by the fact that rundown of channel activity was rarely observed in cell-attached experiments, this patch clamp configuration being unique at providing the appropriate conditions to maintain the channel in a reduced state.

Localization of the SH Groups

The localization of critical SH groups was investigated in patch clamp experiments where the SH modifying agents were applied either at the internal or external surface of patch excised membranes. Because thimerosal is poorly membrane permeable, the fact that thimerosal-dependent K(Ca^{2+}) channel inhibition was observed in inside-out but not outside-out patch-clamp experiments suggest that cytosolic SH groups are involved (Fig. 10). A similar inhibition pattern was observed with DTNB (Fig. 9). The charged groups on DTNB are likely to keep the molecule out of the lipid phase, resulting in a limited lipid solubility. In contrast to thimerosal however, a prolonged application of DTNB at a high concentration (5 mM) was able to induce in outside-out experiments a partial inhibition of channel activity. Because DTNB is slightly more membrane permeable than thimerosal, the DTNB-induced channel inhibition measured under these conditions may have been caused by a cytosolic interaction of DTNB with the channel. The primary structure of the K(Ca^{2+}) channel of intermediate conductance in BAE cells is not currently known. Results derived for the members of the Slo Maxi K(Ca^{2+}) channel family have indicated however that most of the cysteine residues are located in the cytosolic domains of the channel [41]. The data on cysteine distribution in Maxi K(Ca^{2+}) channels therefore supports the results presented in Figs. 9 and 10 where sulfhydryl modifying agents caused a significant inhibition of channel activity when added to the cytoplasmic-like solution only.

Physiological Implications

An overproduction of reactive oxygen species has been associated to an increasing number of endothelial dysfunctions [8, 16, 18, 32, 39]. The evidence gathered to date on endothelial cells shows that a prolonged oxidative stress (15 to 180 min) leads to an inhibition of the capacitative Ca^{2+} influx [44]. Among other possibilities, this inhibition could be the result of a depolarization of the cell potential coming from the closure of K^+ selective channels or the activation of Na^+-selective and/or nonselective cation channels [24]. Evidence has already been reported for a gradual cation channel-mediated depolarization in calf vascular endothelial cells in response to a prolonged oxidative stress [9, 24]. In addition, Fransceschi et al. [13] have shown, that acute exposure of BAE cells to an $O_2\cdot$ generating system induces a hyperpolarization of the cell potential which was transient, despite the generation of a global intracellular Ca^{2+} increase. Our results provided new observations compatible with a cell depolarization consequent to the inhibition of K(Ca^{2+}) channels via the oxidation of critical SH groups. The importance of this phenomenon to the effect of reactive oxygen species on Ca^{2+} homeostasis in endothelial cells remains to be further investigated.

The authors would like to acknowledge the contributions of L. Garneau and of Dr. Lucie Parent and Dr. M.J. Coady who kindly read the manuscript. This work was supported by grants from the Medical Research Council of Canada and the Heart and Stroke Foundation of Canada.

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