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Susceptibility of cloned K^+ channels to reactive oxygen species

(rose bengal/*tert*-butyl hydroperoxide/T lymphocytes/cardiac cells/neurons)

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ABSTRACT **Free radical-induced oxidant stress has been implicated in a number of physiological and pathophysiological states including ischemia and reperfusion-induced dysrhythmia in the heart, apoptosis of T lymphocytes, phagocytosis, and neurodegeneration. We have studied the effects of oxidant stress on the native K^+ channel from T lymphocytes and on K^+ channels cloned from cardiac, brain, and T-lymphocyte cells and expressed in *Xenopus* oocytes. The activity of three Shaker K^+ channels (Kv1.3, Kv1.4, and Kv1.5), one Shaw channel (Kv3.4), and one inward rectifier K^+ channel (IRK3) was drastically inhibited by photoactivation of rose bengal, a classical generator of reactive oxygen species. Other channel types (such as Shaker K^+ channel Kv1.2, Shab channels Kv2.1 and Kv2.2, Shal channel Kv4.1, inward rectifiers IRK1 and ROMK1, and hIsK) were completely resistant to this treatment. On the other hand *tert*-butyl hydroperoxide, another generator of reactive oxygen species, removed the fast inactivation processes of Kv1.4 and Kv3.4 but did not alter other channels. Xanthine/xanthine oxidase system had no effect on all channels studied. Thus, we show that different types of K^+ channels are differently modified by reactive oxygen species, an observation that might be of importance in disease states.**

Molecular oxygen occupies an essential role in many of the metabolic processes associated with an aerobic existence. Oxidation and reduction reactions can lead to the formation of a variety of reactive oxygen species such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH·), or the long-lived diffusible singlet oxygen molecule ($^1\Delta_gO_2$) (1, 2).

These reactive oxygen species have been recently implicated in a variety of physiological and pathophysiological processes (3), such as apoptosis (for review, see ref. 4), cerebral ischemia (5), neurodegeneration (6), myocardial ischemia following reperfusion (7), or with heart failure in the hypertrophic heart (8).

Voltage-sensitive K^+ channels play an essential role in the generation of electrical activity in both neuronal and cardiac cells (9) but are also essential transport systems in a variety of other cell types such as T lymphocytes (10) and renal epithelia (11).

Modifications of K^+ channel activity by reactive oxygen species would lead to drastic changes in the electrical excitability of neuronal and cardiac cells and could easily explain arrhythmias, a tendency to brain hyperexcitability, or even neuronal death.

Tremendous progress has recently been made in the molecular biology of K^+ channels. The first class of cloned mammalian K^+ channel genes corresponds to proteins with six transmembrane segments and represents the voltage-dependent outward rectifier class (for reviews, see refs. 12–14). The second class of cloned K^+ channels corresponds to proteins with two transmembrane domains and represents the class of inward rectifier K^+ channels (for reviews, see refs. 15 and 16). The third class of protein structure that generates a slow K^+ channel activity in *Xenopus* oocytes presently includes a single representative, called IsK, characterized by a single transmembrane domain (for review, see ref. 17).

Several studies have reported the possibility that reactive oxygen species alter ionic channel function (18–20). In the present report, we have investigated the effects of several reactive oxygen species generating systems on members of the three structural classes K^+ channels expressed in *Xenopus* oocyte.

MATERIALS AND METHODS

mRNA Isolation and Functional Expression in *Xenopus* Oocytes. DNA sequences coding for Kv4.1 (21), Kir2.1/IRK1 (22), and Kir1.1a/ROMK1 (23) channels were amplified by PCR with a low-error-rate DNA polymerase (*Pfu* DNA polymerase, Stratagene) and subcloned into pEXO (24) or pBTG vectors (25). Syntheses of corresponding complementary RNAs were carried out *in vitro* with T7 or T3 RNA polymerase (Stratagene). On the other hand, cloning of cDNA and synthesis of complementary RNA have been described for Kv1.2/RCK5 (26), Kv1.3/HLK3 (27), Kv1.4/RHK1 (28), Kv1.5 (29), Kv2.1/drk1 (30), Kv2.2/cdrk (31), Kv3.4/Raw3 (32), Kir2.3/IRK3 (33), and hIsK (27) channels. Preparation of oocytes and mRNA injection have been described (26).

Solutions. A rose bengal (Sigma) stock solution was made daily in ND96 saline solution (26) at the concentration of 1 mM and kept at 4°C in darkness because of a strong decay in the activity of the dye in light. Diluted rose bengal solutions were renewed prior to each experiment and kept in darkness. *tert*-Butyl hydroperoxide (tBHP) solutions were made daily from a 70% (wt/vol) commercial solution (Sigma). Stock solutions of diethylenetriaminepentaacetic acid and xanthine were prepared daily at the concentration of 1 mM in ND96 solutions and kept at room temperature. A stock solution of xanthine oxidase (1 international unit/ml) in water was kept at 4°C. Diethylenetriamine pentaacetic acid (50 μM), xanthine (50 μM), and xanthine oxidase (50×10^{-3} international unit/ml) were mixed prior to each experiment. The 2 mM histidine stock solution (Sigma) was made daily in ND96 solution and kept at 4°C. A stock solution of Cu^{2+}/Zn^{2+} superoxide dismutase (Sigma) was made in water at 1000 international units/ml and kept at 4°C.

Electrophysiological Measurements in *Xenopus* Oocytes. Microelectrode procedures have been described (26). The experimental chamber was illuminated with a broad-band white light (150 W, GLI 154, Fort, France) equipped with optic fibers that delivered ≈1200 lux at 533 ± 10 nm. Drugs were applied externally by addition to the superfusate (flow rate, 3 ml/min). The control saline solution ND96 contained 2 mM

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Abbreviations: tBHP, *tert*-butyl hydroperoxide; *I–V*, current–voltage.
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K^+. To study inward rectifier K^+ channels, a K^+-rich solution (98 mM K^+) solution was used. In some experiments, ND96 was bubbled with pure oxygen or nitrogen for at least 1 h before experiments were started and then continuously gassed throughout the experiments. All experiments were performed at room temperature (21–22°C). The variability of the results was expressed as the SEM with n indicating the number of oocytes contributing to the mean.

Patch-Clamp Recordings. Voltage-clamp experiments were performed on the human T-lymphocyte cell line Jurkat. A human B-lymphocyte cell line (IM9) permanently transfected with the Kv1.3 channel was also used (34). The whole cell and the outside-out configurations of the patch-clamp technique were used as described (34). The external solution contained 117.5 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.2 adjusted with NaOH). The internal solution contained 115 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.2 adjusted with KOH). By using a microperfusion system, the medium bathing a single cell could be exchanged in <5 sec.

RESULTS

The effect of the various reactive oxygen species generating systems was first studied in control noninjected oocytes. Photoactivation of rose bengal was used to produce reactive oxygen species (35). Rose bengal was first superfused for 2.5 min in darkness (Fig. 1*A*). Then the oocyte was illuminated for 5 min in the continuous presence of rose bengal. Fig. 1*B* shows current–voltage (*I–V*) curves elicited by voltage ramps in oocytes superfused with increasing concentrations of rose bengal after 5 min of photoactivation. Rose bengal induced a linear current between −100 mV and +50 mV that reversed at −10.9 ± 1.4 mV (n = 15). The amplitude of the rose bengal-induced current increased in a dose-dependent manner. Fig. 1*C* shows that rose bengal induced a current only during photoactivation; the slight increase of the current in dark conditions was not significant (Student's t test, P = 0.12). This current stabilized when illumination was stopped. The effect of rose bengal was not reversible after a 1-h wash period. However, an overnight wash in the dark completely reversed the rose bengal-induced current activation (data not shown). The effect of rose bengal in light conditions was enhanced in the presence of pure oxygen in the saline solution and was conversely decreased in the presence of pure nitrogen (Fig. 1*D*). The ionic selectivity of the rose bengal-induced current was determined. When most of the chloride was replaced with gluconate (9.6 mM instead of 105.6 mM Cl^-), the reversal potential (−11.7 ± 2.9 mV, n = 4) was not significantly different from control conditions. When external sodium was replaced with *N*-methyl-D-glucamine, the reversal potential shifted toward hyperpolarizing values (−35.9 ± 3.5 mV, n = 5). Conversely, when sodium was replaced with potassium (98 mM K^+), the reversal potential was slightly shifted toward depolarizing values (−5.6 ± 3.4 mV, n = 3). These results suggested that the rose bengal-induced current is probably due to a cationic channel.

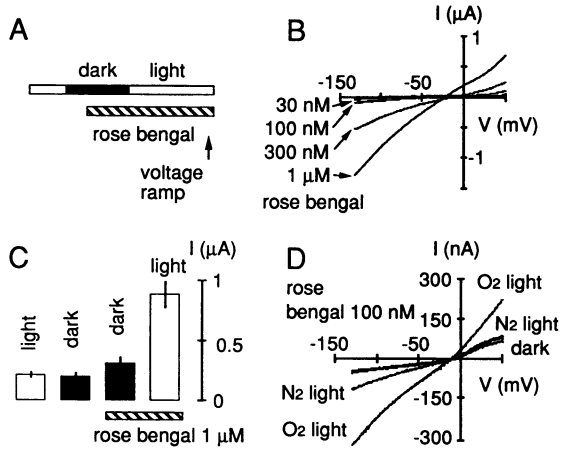

FIG. 1. (*A*) Experimental protocol used to photoactivate rose bengal. (*B*) Averaged *I–V* curves elicited in control oocytes by voltage ramps (1 s in duration) from −130 mV to +50 mV after 5 min of photoactivation in the presence of increasing concentrations of rose bengal (n = 3). (*C*) Current amplitude recorded at +50 mV in control and in the presence of 1 μM rose bengal in dark and light conditions (n = 3). (*D*) Averaged *I–V* curves recorded during oxygen (n = 6) or nitrogen (n = 7) bubbling in the presence of 100 nM rose bengal, before and after 5 min of illumination. (*E*) Cloned K^+ channels sensitive and resistant to 100 nM photoactivated rose bengal.

The effect of 100 nM rose bengal before and after photoactivation was studied in oocytes expressing various types of cloned K^+ channels including three species of inward rectifiers (IRK1, IRK3, and ROMK1), four Shaker (Kv1.2, Kv1.3, Kv1.4,

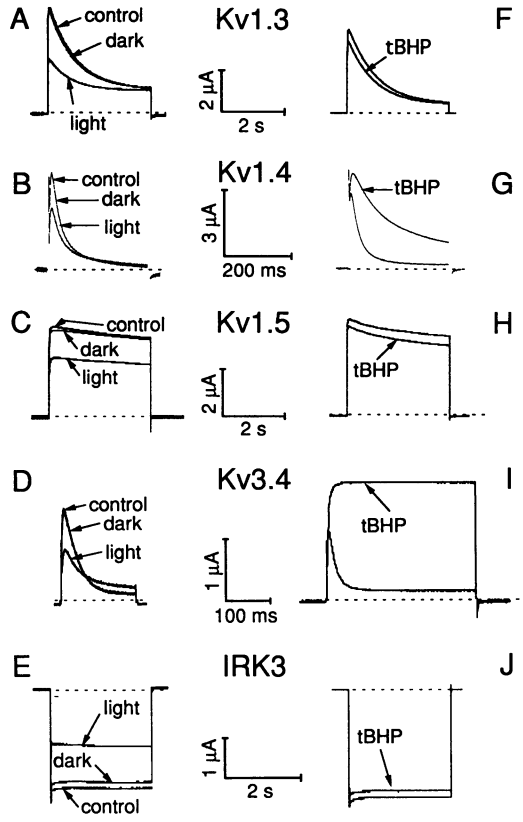

FIG. 2. Effect of 100 nM rose bengal on Kv1.3 (*A*), Kv1.4 (*B*), Kv1.5 (*C*), Kv3.4 (*D*), and IRK3 (*E*) and effect of 1 mM tBHP on Kv1.3 (*F*), Kv1.4 (*G*), Kv1.5 (*H*), Kv3.4 (*I*), and IRK3 (*J*). Kv1.3, Kv1.4, Kv1.5, and Kv3.4 were recorded during voltage pulses to +30 mV from a holding potential of −80 mV. IRK3 was recorded during voltage pulses to −80 mV from a holding potential of 0 mV in a K^+-rich solution.

tBHP did not affect endogenous conductances in control oocytes (n = 6; data not shown). The xanthine/xanthine oxidase system (36) was without effect in most of the batches but induced a Ca^{2+}-dependent Cl^- current in some batches (data not shown).

The effect of 100 nM rose bengal before and after photoactivation was studied in oocytes expressing various types of cloned K^+ channels including three species of inward rectifiers (IRK1, IRK3, and ROMK1), four Shaker (Kv1.2, Kv1.3, Kv1.4,

and Kv1.5), two Shab (Kv2.1 and Kv2.2), one Shaw (Kv3.4), one Shal (Kv4.1), and the IsK protein (hIsK). Oocytes were first superfused for 2.5 min with 100 nM rose bengal in the dark, and then the dye was photoactivated for 5 min. The activity of Kv1.3 (n = 20), Kv1.4 (n = 9), Kv1.5 (n = 8), Kv3.4 (n = 8), and IRK3 (n = 9) channels was inhibited by >30% after 5 min of illumination in the presence of 100 nM rose bengal (Figs. 1*E*, 2, and 3). The expression of K^+ channels hIsK (n = 4), Kv1.2 (n = 5), Kv2.1 (n = 3), Kv2.2 (n = 3), Kv4.1 (n = 5), IRK1 (n = 4), and ROMK1 (n = 4) was insensitive to the above treatment (<10% inhibition) (Fig. 1*E*).

When the illumination in the presence of 100 nM rose bengal lasted >5 min, K^+ currents for all the sensitive channels were further inhibited to >80%. The intensity of K^+ currents stabilized when illumination stopped (data not shown). Washing oocytes to remove rose bengal in dark conditions for an hour did not reverse the inhibitory effect (data not shown).

The effects of photoactivated rose bengal were compared with those of tBHP, another generator of reactive oxygen species (Fig. 2 and Fig. 3). tBHP did not have any significant effect on Kv1.3, Kv1.5, and IRK3 channels (Figs. 2 *F*, *H*, and *J* and 3). However, it produced a drastic increase of the amplitude of the Kv3.4 current due to the removal of the fast inactivation process (n = 4; Figs. 2*I* and 3). The same type of effects were observed with the rapidly inactivating Kv1.4 channel with a severe decrease of the inactivation rate (time constant from 35.0 ± 1.9 ms to 93.6 ± 3.9 ms, n = 7; Figs. 2*G* and 3).

Fig. 3 shows that xanthine/xanthine oxidase did not affect the rose bengal-sensitive K^+ channels. Fig. 4 shows that tBHP and rose bengal have an additive effect on Kv1.4 and Kv3.4. Photoactivation of rose bengal in the continuous presence of tBHP produced an inhibition of the Kv1.4 and Kv3.4 currents with no significant alteration in their inactivation kinetics. The effect of tBHP on Kv3.4 activity was completely reversible on washing (data not shown).

The effect of photoactivated rose bengal on the activation/inactivation parameters of the Kv1.3 channel was studied in more detail. Neither the membrane potential for half-activation ($V_{0.5}$ = −15.5 mV) or half-inactivation ($V_{0.5}$ = −28.6 mV) nor the slope factors k = 7 mV (activation) and k = 3.8 mV (inactivation) were modified by photoactivated rose bengal (data not shown).

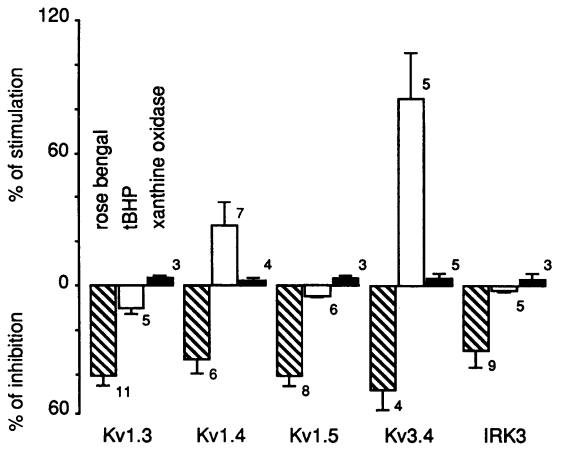

FIG. 3. Amplitude histogram showing the effect of 100 nM photoactivated rose bengal, 1 mM tBHP, and xanthine oxidase (50×10^{-3} international unit) on Kv1.3, Kv1.4, Kv1.5, Kv3.4, and IRK3. Each oxygen radical generating system was applied for 5 min. Numbers of oocytes tested are indicated by the bars. Kv1.3, Kv1.4, Kv1.5, and Kv3.4 were recorded during voltage pulses to +30 mV from a holding potential of −80 mV. IRK3 was recorded during voltage pulses to −80 mV from a holding potential of 0 mV in a K^+-rich solution. The endogenous current activated by rose bengal was subtracted from the exogenous current by assuming a linear I–V curve between −100 mV and +50 mV. Bars: hatched, rose bengal; open, tBHP; solid, xanthine oxidase.

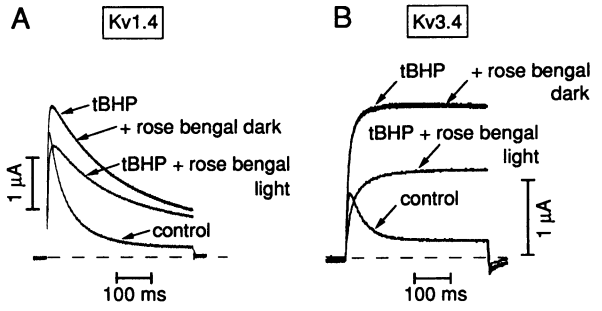

FIG. 4. Additive effect of both 1 mM tBHP and 100 nM photoactivated rose bengal on Kv1.4 (*A*) and Kv3.4 (*B*). In *A* and *B*, the holding potential was −80 mV and the test potential was +30 mV.

In aqueous solution, rose bengal is elevated to its triplet state by illumination (at 500–600 nm). In the presence of dissolved oxygen, the decay of the rose bengal triplet generates singlet oxygen (75% of decays) and superoxide (20% of decays) (35). Fig. 5*A* shows that oxygen bubbling compared to nitrogen bubbling significantly enhanced Kv1.3 inhibition (n = 6). Experiments have been carried out to try to establish which of the reactive species produced by photoactivation of rose bengal is responsible for the observed effects. In the presence of histidine, which reacts about 10^8 times more rapidly with oxygen singlet than with superoxide (2), photoactivation of rose bengal failed to produce any significant inhibition (n = 5; Fig. 5*B*). Conversely, Cu^{2+}/Zn^{2+} superoxide dismutase, which should eliminate superoxide production, did not provide protection against photoactivated rose bengal (Fig. 5*B*).

Kv1.3 is the major K^+ channel in human T lymphocytes (27). Kv1.3 was recorded in Jurkat T lymphocytes by using the whole cell configuration of the patch-clamp technique. Considering that the Kv1.3 current is not sensitive to internal ATP and slightly depressed by internal Ca^{2+} (27), the Jurkat T cells were dialyzed with an internal medium lacking free Ca^{2+} and ATP. The application of rose bengal (100 nM) in the dark produced a slight inhibition of the K^+ current elicited during a depolarization to +30 mV (Fig. 6 *A* and *B*). During photoactivation, rose bengal inhibited the outward current by 89 ± 9% (n = 8) within 2 min (Fig. 6 *A*–*C*). Similar results with the human B-lymphocyte cell line IM9 transfected with Kv1.3 were obtained by using the patch-clamp whole-cell (data not shown) and outside-out configurations (Fig. 6*D*).

DISCUSSION

Reactive oxygen species form a family of highly reactive molecules including superoxide (O_2^-), hydroxyl radical (OH·),

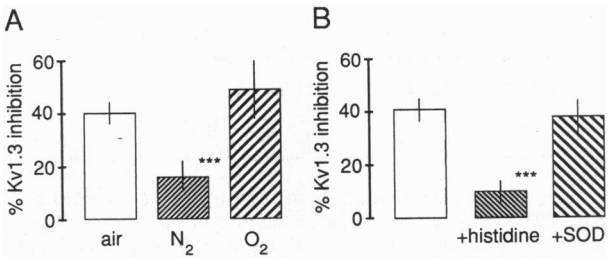

FIG. 5. (*A*) Effect of 100 nM rose bengal on Kv1.3 in air, nitrogen, and oxygen saturated solutions in light (n = 6). (*B*) Effect of 500 μM histidine and superoxide dismutase (SOD, microinjected intracellularly at 50×10^{-9} international unit per oocyte) on current inhibition induced by 100 nM photoactivated rose bengal (5 min). ***Confidence limit of 0.001 (Student's t test).

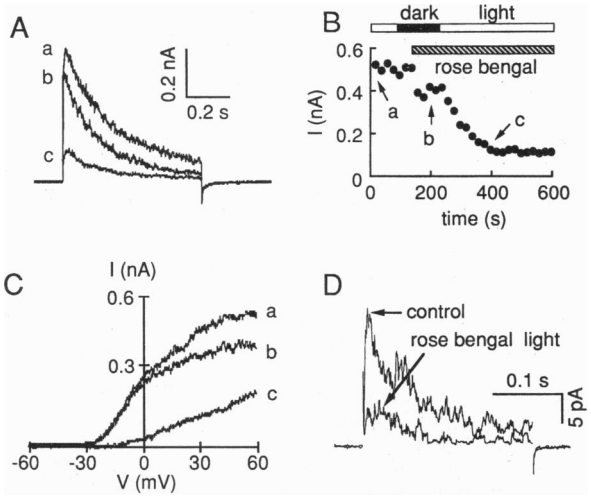

FIG. 6. (*A–C*) Effect of 100 nM photoactivated rose bengal on K^+ currents recorded with the whole cell configuration in Jurkat T lymphocytes. (*A*) Current traces recorded during voltage steps to +30 mV from a holding potential of −80 mV. (*B*) Time course of the effects of rose bengal on current amplitude. (*C*) Steady-state *I–V* curves measured during voltage ramps (500 ms in duration) from a holding potential of −100 mV. Traces a–c indicate time of recording shown in *B*. (*D*) Effect of 100 nM photoactivated rose bengal on K^+ currents recorded with the outside-out configuration in IM9 B lymphocytes permanently transfected with Kv1.3. The holding potential was −80 mV and the test pulse was +30 mV.

and hydrogen peroxide (H_2O_2), which are encountered in many biological systems (for review, see ref. 3). Singlet oxygen molecule ($^1\Delta_gO_2$) is also a very reactive oxygen species (2). It is produced in phagocytosing neutrophiles (37) when hypochlorite (OCl^-), formed in the myeloperoxidase reaction, reacts with H_2O_2 (1). It is also produced in the reaction between peroxinitrite and hydrogen peroxide (38). In the presence of dissolved oxygen, many molecules such as porphyrins and xanthenes (rose bengal), acting as sensitizers, generate $^1\Delta_gO_2$ upon sufficient illumination (2).

Exposure of hearts to photoactivated rose bengal has been shown to lead to electrocardiographic abnormalities and arrhythmias (39), while histidine can significantly reduce arrhythmias after ischemia–reperfusion in isolated perfused hearts (40). Photoactivated rose bengal inhibits the delayed rectifier K^+ current and activates leakage currents in frog cardiac cells (18, 41). It inhibits an inward rectifier K^+ current and activates a calcium-activated cationic current in rabbit ventricular cells (19). A similar photoactivated rose bengal-induced cationic current has been shown by Tarr *et al.* (42) in frog cardiac cells. Moreover, Kuo *et al.* (41) have suggested that the early cellular response to oxidative stress in nonexcitatory cells is the activation of K^+ channels. In isolated dopaminergic substantia nigra neurones, an oxidative stress produced with tBHP reduced the fast inactivation of an A-type K^+ current (43). In addition it was recently shown (20) that the fast inactivation of cloned mammalian A-type K^+ channels including Kv1.4 and Kv3.4 expressed in *Xenopus* oocyte is abolished by oxidation and maintained by reducing agents like glutathione. Finally, photomodifications of squid axons produce a prolongation of action potentials due to a modification of K^+ and Na^+ currents (44) and H_2O_2 has been shown to inhibit the fast inactivation of Kv3.3 and Kv3.4 K^+ channels (45).

The aim of the present study was to identify which of the cloned K^+ channels are sensitive to various reactive oxygen generating systems. The activities of three Shaker channels (Kv1.3, Kv1.4, and Kv1.5), one Shaw channel (Kv3.4), and one inward rectifier (IRK3) were drastically depressed when rose bengal was photoactivated in a normal air atmosphere. The percentage of channel inhibition was decreased when solutions were gassed with pure nitrogen while it was enhanced when they were gassed with pure oxygen. This result indicates that channel inhibition is due to the production of reactive oxygen species rather than to a direct effect of photoactivated rose bengal on K^+ channels. The effects due to the photoillumination of rose bengal were compared with those of tBHP, which is known to produce predominantly hydroxyl radicals (2). tBHP reversibly enhanced the activity of Kv1.4 and Kv3.4 by attenuating or removing their fast inactivation processes. tBHP-stimulated Kv1.4 and Kv3.4 currents could still be inhibited by photoactivation of rose bengal. tBHP did not affect the activity of Kv1.3, Kv1.5, and IRK3 channels. These results show that various systems generating reactive oxygen species produce different effects on the susceptible K^+ channels. The effects of xanthine/xanthine oxidase on rose bengal-sensitive channels were then studied in solutions bubbled with pure oxygen. Under *in vitro* conditions, xanthine oxidase catalyzes the oxidation of xanthine and produces mainly superoxide radicals in elevated P_{O_2} and in the presence of diethylenetriaminepentaacetic acid, which minimizes production of hydroxyl radicals (36). No effect of this reactive oxygen species generating system was found on K^+ channels. The observations (*i*) that histidine significantly reduced the effects of rose bengal, (*ii*) that intracellular microinjection of superoxide dismutase, which eliminates intracellular superoxide species, failed to have a protective effect, and (*iii*) that the xanthine/xanthine oxidase system was without effect suggest that inhibition by photoactivated rose bengal is likely not due to the production of superoxides. Nevertheless, it should be kept in mind that in each generating system the designated compounds can probably initiate chain reactions that produce additional potentially toxic radicals that could be involved in the recorded effects. Thus, it remains difficult to definitely assess the relative importance of each reactive oxygen species.

Closely related channels of the Shaker or of the inward rectifier family strongly differ in their susceptibility to photoactivated rose bengal. Since some amino acids (histidine, tryptophane, cysteine, and methionine) are preferentially modified by reactive oxygen species, especially by oxygen singlets (2), sequence alignments have been performed and revealed interesting differences. For example, the sensitive channel IRK3 contains histidine (positions 2, 4, 11, 189, 329, and 421), cysteine (positions 79 and 346), and methionine (positions 43, 60, 115, 166, 227, 273, and 427) residues that are absent in the resistant channel IRK1 at the corresponding positions. One or more of these residues might be implicated in the sensitivity of the IRK3 channel to reactive oxygen species.

The effects of photoactivated rose bengal on the biophysical properties of Kv1.3 have been investigated with more details. Photoactivated rose bengal reduces the number of functional Kv1.3 channels with no change of activation or inactivation parameters. The cloned channels expressed in *Xenopus* oocytes are in a different environment compared to the same channels expressed in their normal cellular host. However, the antioxidant capabilities of the oocyte may not be designed to protect ion channels while native cells may have such capabilities. Since Kv1.3 is the major channel expressed in human T lymphocytes, it was then important to analyze the effect of reactive oxygen species directly on this cell type. K^+ channel currents recorded in Jurkat T lymphocytes and in transfected IM9 B lymphocytes were drastically decreased by photoactivation of rose bengal, as they were when expressed in oocytes. The inhibition recorded in the outside-out configuration in the transfected IM9 cell line indicates a direct effect of reactive oxygen species on Kv1.3 channels.

In summary, the present work identifies two effects of reactive oxygen species on cloned K^+ channels. Photoactivated

rose bengal leads to the inhibition of particular K^+ channel types, such as Kv1.3, the major channel in T lymphocytes, and the cardiac Kv1.4 and Kv1.5 channels, whereas tBHP impairs the fast inactivation process of Kv3.4 and the ubiquitous (cardiac/neuronal) Kv1.4 channel. Since apoptosis of T lymphocytes occurring during thymic selection involves reactive oxygen species, the present results suggest that Kv1.3 might possibly be inhibited during programmed T-cell death. On the other hand, Kv1.4, Kv1.5, and IRK3 channels are expressed in cardiac cells (28, 29, 33). The Kv1.4 and Kv1.5 are the fast inactivating cardiac channels, whereas IRK3 is the major cardiac delayed rectifier channel, and the latter is a crucial inward rectifier. Therefore, it is probable that the action of reactive oxygen species on K^+ channels will contribute to the major electrophysiological disorders that occur during reperfusion-induced arrhythmias after an ischemia and during heart failure induced by chronic pressure overload (8). The same channels are present in the brain and their inhibition by reactive oxygen species might play a role in changes in electrical identity of neurons produced by ischemia and of course in neuronal death.

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