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EFFECTS OF H_2O_2 ON MEMBRANE POTENTIAL AND $[Ca^{2+}]_i$ OF CULTURED RAT ARTERIAL SMOOTH MUSCLE CELLS

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Summary. The effect of 1 mmol/l H_2O_2 was studied on the membrane potential and $[Ca^{2+}]_i$ with microelectrodes and the fura-2 technique, respectively. H_2O_2 induced a biphasic increase in $[Ca^{2+}]_i$ with a fast transient peak and a subsequent plateau. H_2O_2 also led to a biphasic hyperpolarization of the cells with a similar time course. This was followed by a slight depolarization after wash-out of H_2O_2. External Ca^{2+} free solutions and treatment with the Ca^{2+} ionophore A23187 (1 µmol/l) abolished the effect of H_2O_2 on $[Ca^{2+}]_i$ and almost entirely reduced the effect on the membrane potential. Phenylephrine (10 µmol/l) or A23187 also induced very similar biphasic hyperpolarizations of the membrane as H_2O_2 which were fully reversible after wash-out. It is concluded that H_2O_2 hyperpolarizes the membrane by opening of Ca^{2+} dependent K^+ channels.

In the recent years the concept has been put forward that reactive oxygen species (ROS) contribute to the plaque development in atherosclerosis [1-3]. H_2O_2 which is produced by activated macrophages, but also to a lesser extent by the endothelium [4] belongs to this group of reactive oxygen species. It has been reported that H_2O_2 stimulates cell growth and proto-oncogene expression in SMCs similarly to growth factors [5]. In some studies it has been found that reactive oxygen species also have vasoconstrictor properties like growth factors [6,7]. However, in other studies relaxation of smooth muscle cells has been observed [8,9]. Since different mechanisms may underly relaxation and contraction [8] we tested in the present study whether H_2O_2 influences the membrane potential of SMCs which could explain alterations in muscle tone as it has been shown in a previous study for nitric oxide [10]. Several studies demonstrate that oxidative stress influences ion channel activity and function in a variety of different cell types such as skeletal muscle cells [11], neurons [12], lung adenocarcinoma cells [13], liver cells [14] and pancreatic B-cells [15-18]. Evidently, the mechanisms by which H_2O_2 and specific SH-group reagents influence ion channels and cell function are different [16,17]. The results presented in this paper suggest that H_2O_2 increases $[Ca^{2+}]_i$ and thereby activates the Ca^{2+}-dependent K^+ channel in arterial smooth muscle cells.

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MATERIALS AND METHODS

Smooth muscle cells were taken from a cell culture of rat carotis arteries established at the pharma research laboratories of Hoffmann-La Roche Ltd. (Basel, Switzerland). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), DMEM/F12=4:1 with 10 % fetal calf serum (FCS) and standard penicillin-streptomycin at 37° C and 5 % CO_2. Cells were used since passage number 17.

The membrane potential difference was recorded with reference to the grounded bath using conventional microelectrodes as described previously [19]. The electrodes had input resistances of 100-200 MΩ when filled with 1 mol/l KCl. The cells were continuously superfused with a bath solution containing (in mmol/l): NaCl 115, KCl 5, $CaCl_2$ 1.3, $MgCl_2$ 1, $NaHCO_3$ 21, NaH_2PO_4 2, glucose 5, bubbled with a gas mixture of O_2/CO_2 95/5 % to maintain a pH of 7.4 at 37 °C. Ca^{2+} free salines contained 1 mmol/l EGTA and no Ca^{2+}. Reagents were added to the bath solution.

Intracellular free calcium ($[Ca^{2+}]_i$) was determined using the fluorescent dye fura-2 (fura-2AM, Molecular Probes, Eugene, OR, USA). Cells were incubated in 10 µmol/l fura-2AM for 45 min, followed by a 30 min incubation in cell culture media. Measurements were made by fluorescence microspectrophotometry. Light of alternating excitation wavelength (340/380nm) from a monochromator light source (Uhl, München, FRG) was deflected into the microscope objective. Emitted fluorescence was directed through a 475nm cut-off filter to the photomultiplier tube (213-IP28A, Seefelder Meßtechnik, Seefeld, FRG). Fluorescence in the absence of dye was less than 6 % of that in dye-loaded cells and was not significantly modified by the experimental manoeuvres, ruling out optical interference by autofluorescence and scattered light. Dye calibration was made according to the method established by Grynkiewicz *et al.* [20] for determination of individual $[Ca^{2+}]_i$ values.

Phenylephrine and A23187 were from Sigma (Deisenhofen, FRG). Charybdotoxin was from Bachem (Heidelberg, FRG). DMEM and FCS were from Gibco (Eggenstein, FRG). All other chemicals were from Merck, Darmstadt, FRG in the purest grade available.

Experiments are illustrated by representative recordings or are presented as means ± SEM for the indicated number of experiments (n). Statistical significance was accepted for $P \leq 0.05$ (Student's t-test).

RESULTS

Effects of H_2O_2 on membrane potential and $[Ca^{2+}]_i$

The effect of H_2O_2 on the membrane potential (MP) of cultured rat carotis smooth muscle cells (SMC) is shown in figure 1 for one representative experiment. The control MP was -44±3 mV (n=5) in this series of experiments. Addition of H_2O_2 (1 mmol/l) led to a biphasic hyperpolarization with a fast transient to -64±2 mV (n=5) followed by a plateau with a mean value of -52±3 mV (n=5). After wash-out of H_2O_2 the MP was slightly depolarized as compared to controls (-39±4 mV, n=4). In two cells the depolarization started already in the presence of H_2O_2. H_2O_2 also affected the intracellular free Ca^{2+}-concentration ($[Ca^{2+}]_i$) and this was again a biphasic response (Fig. 2). The relative fluorescence of fura-2 expressed as the ratio F340/F380 was 0.51±0.04 (97±15 nmol/l Ca^{2+}) (n=5) on average under control conditions. After addition of 1 mmol/l H_2O_2 it increased with a similar time course as the MP to a transient peak of 0.74±0.09 (192±41 nmol/l Ca^{2+}) (n=5) and then declined to a plateau with an average ratio of 0.65±0.07 (151±28 nmol/l Ca^{2+}) (n=5). This effect was not reversible after wash-out of H_2O_2 (not shown), but the addition of 5 mmol/l of the reducing agent dithioerythritol (DTE) decreased the ratio to control values (0.59±0.03 (126±12 nmol/l Ca^{2+}), n=3). The similarity in the time course of the effect of H_2O_2 on the MP and $[Ca^{2+}]_i$ led to the supposition that the hyperpolarization is owing to the opening of Ca^{2+} dependent K^+ channels (K^+_{Ca} channels). Therefore H_2O_2 was tested in Ca^{2+} free salines and in the

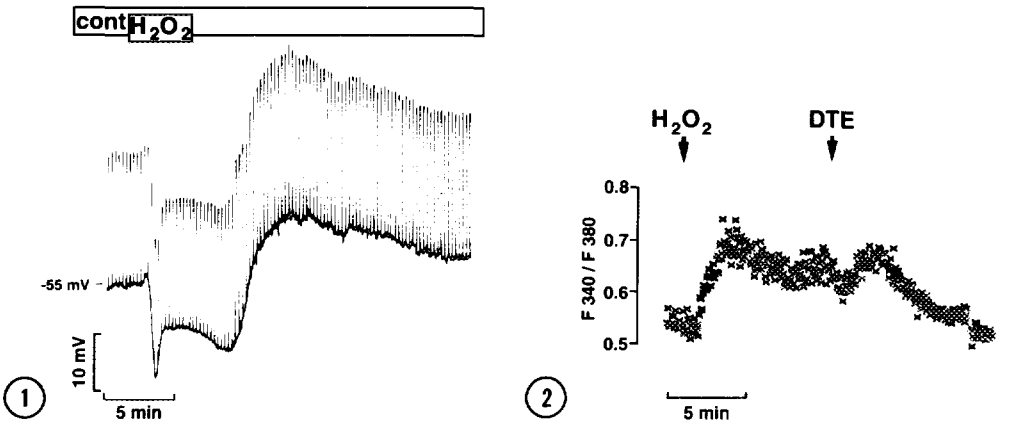

Figure 1. Effects of H_2O_2 (1 mmol/l) on the membrane potential of a single rat carotis smooth muscle cell (SMC). The cell is slightly depolarized after wash-out of H_2O_2. One representative experiment out of 5.
The vertical (upward) voltage deflections are owing to current (65 nA) injections into the microelectrode, thus representing the input resistance (R_{in}). This is also valid for figures 3, 5, and 6.

Figure 2. Effect of H_2O_2 (1 mmol/l) and subsequent addition of dithioerythritol (DTE, 5 mmol/l) on the intracellular free Ca^{2+} concentration measured with fura-2. The experiment is representative of 5 with similar results.
The vertical scale shows the ratio of the fluorescence at the excitation wavelength of 340 nm and 380 nm (also valid for Fig. 4).

additional presence of the Ca^{2+} ionophore A23187 which was used to empty all intracellular Ca^{2+} stores [21]. The control MP in this series of experiments was -58±2 mV (n=4). Exposure of the cells to Ca^{2+} free saline led to a strong depolarization (Fig. 3). The addition of A23187 (1 µmol/l) led to a fast transient hyperpolarization of 23±5 mV (n=4) and the subsequent addition of H_2O_2 (1 mmol/l) slightly and transiently hyperpolarized the membrane by 5±2 mV (n=4). Exposure to Ca^{2+} free solutions did not change $[Ca^{2+}]_i$, the ratios F340/F380 being 0.62±0.04 (205±18 nmol/l Ca^{2+}) and 0.59±0.06 (194±26 nmol/l Ca^{2+}) under control and Ca^{2+} free conditions, respectively. Addition of A23187 dropped the ratio F340/F380 to minimum values (0.12±0.01) which is supposed to be zero $[Ca^{2+}]_i$. The horizontal arrow (Fig. 4) points to a very fast transient in the signal which may represent the emptying of intracellular Ca^{2+} stores, but was too fast to be fully resolved. H_2O_2 did not change $[Ca^{2+}]_i$ under these conditions (Fig. 4).

Effects of phenylephrine and A23187 on membrane potential (MP)

To further elucidate the role of $[Ca^{2+}]_i$ on the membrane potential phenylephrine (PE) and A23187 were tested which both increase $[Ca^{2+}]_i$ [21,22]. Both substances hyperpolarized the MP biphasically in a very similar manner as H_2O_2 (Figs. 5 and 6). On average, the MP increased from -45±3 mV (control, n=7) with PE (10 µmol/l) transiently to -70±2 mV (n=7) and decreased thereafter to a steady state value of -62±4 mV (n=7). The effect was totally reversible. The MP after wash-out of PE was -44±4 mV (n=5). The α_1-adrenoceptor antago-

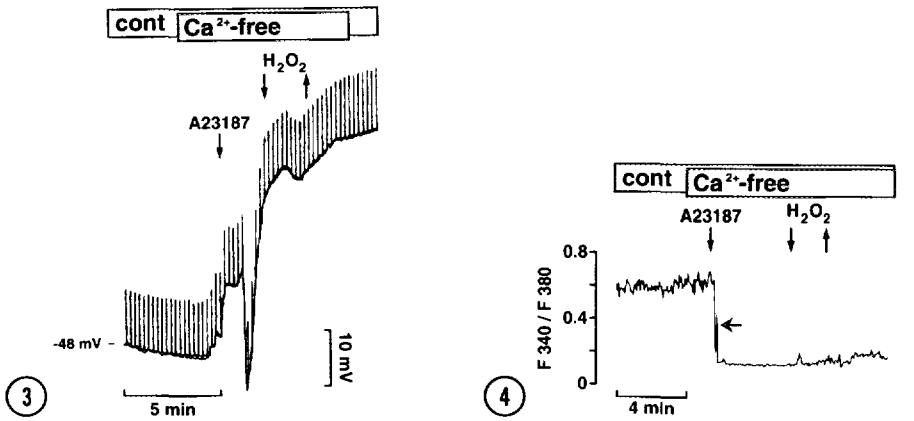

Figure 3. Effect of extracellular Ca^{2+} free solution (1mmol/l EGTA), A23187 (1 µmol/l), and H_2O_2 (1 mmoll/l) on the membrane potential of a single SMC. One representative experiment out of 4.

Figure 4. Effect of extracellular Ca^{2+} free solution (1mmol/l EGTA), A23187 (1 µmol/l), and H_2O_2 (1 mmoll/l) on $[Ca^{2+}]_i$. One representative experiment out of 5.

nist prazosin (10 µmol/l) inhibited the PE elicited response (not shown). There was no direct effect of prazosin on MP the values being -38±3 mV and -39±3 mV (n=4) before and after additon of prazosin, respectively. With prazosin the initial peak response to PE (10 µmol/l) was totally abolished, however, the MP slightly increased on average to -51±3 mV (n=4). With A23187 the control MP was -50±6 mV (n=5) in this series of experiments. It was increased by A23187 (1 µmol/l) to -79±3 (n=5) mV and -66±5 mV (n=5) for the initial transient and steady state MP, respectively. The effect of A23187 was reversible, the MP de-

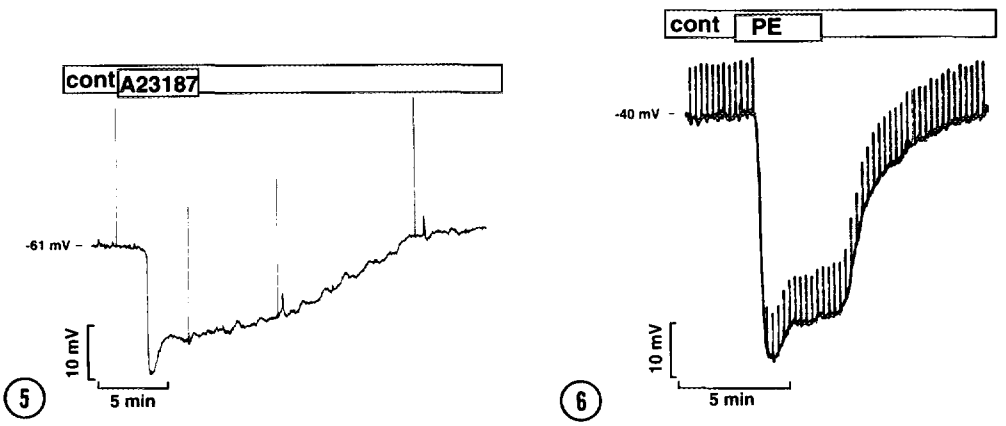

Figure 5. Effect of the Ca^{2+} ionophor A23187 (1 µmol/l) on the membrane potential of a single SMC. This experiment is representative of 5 with similar results.

Figure 6. Effect of phenylephrine (PE, 10 µmol/l) on the membrane potential of a single SMC. One representative experiment out of 7.

clined to -56±8 mV (n=4). To further elucidate whether this effect was owing to an opening of K^+_{Ca} channels A23187 was tested in the presence of charybdotoxin (CTX, 20 nmol/l) (not shown). CTX itself did not influence the MP which was -47±4 mV (n=8) under control conditions and -46±4 mV (n=8) after addition of CTX. After subsequent addition of A23187 a slight transient increase in MP was detectable in 4 out of 8 experiments, however, on average the MP was -50±5 mV (n=8) and not significantly altered. Thereafter the MP was stable at -47±4 mV (n=8) which was not different from control conditions. Therefore, the specific K^+_{Ca} channel blocker CTX almost entirely inhibited the effect of A23187. However, CTX only partly and occasionally affected the H_2O_2 induced hyperpolarization (not shown). Since CTX possess several cysteine residues (and therefore SH-groups) the loss of effectiveness may be owing to a destruction of CTX by H_2O_2.

DISCUSSION

In the present paper it is reported that H_2O_2 similarly to the α_1-agonist phenylephrine and the Ca^{2+} ionophore A23187 hyperpolarizes the membrane potential of cultured smooth muscle cells. We could show that the H_2O_2 induced hyperpolarization was also accompanied by an increase in the intracellular Ca^{2+} concentration. An increase of $[Ca^{2+}]_i$ after treatment of SMCs with H_2O_2 is in agreement with observations reported in other studies which show that this augmentation is at least partly due to mobilization of Ca^{2+} from intracellular stores [23,24]. Increasing $[Ca^{2+}]_i$ by means of the Ca^{2+} ionophore A23187 induced a hyperpolarization which was almost completely blocked by the specific inhibitor of Ca^{2+} dependent K^+ channels, charybdotoxin [25,26]. Moreover, the effect of H_2O_2 on MP and $[Ca^{2+}]_i$ was almost entirely blocked or abolished in Ca^{2+} free solutions (containing A23187) and thus H_2O_2 seems indeed to hyperpolarize the membrane via K^+_{Ca} channels present in these cells. The existence of the Ca^{2+}-dependent K^+ channel in smooth muscle cells has been demonstrated in cell-attached patch-clamp recordings on rabbit aorta cells [27].

It is commonly accepted that H_2O_2 which is generated *in vivo* by macrophages and endothelial cells influences smooth muscle cell function. However, the effects of H_2O_2 on smooth muscle cells are inconsistent. It has been shown that H_2O_2 induces vasoconstriction in smooth muscle cells [7,28] as well as relaxation [8,9,29]. There are several reasons for this discrepancy. H_2O_2 seems to differently affect the function of the same kind of smooth muscle cells in distinct species [8]. It has been shown that H_2O_2 contracts smooth muscles at low concentrations and relaxes them in the same preparation at higher concentrations [7]. Moreover, it is not unlikely that the action of H_2O_2 varies in smooth muscles of different tissues.

The hyperpolarization observed in this study with cultured smooth muscle cells could explain muscle relaxation. This is consistent with the observation on pig coronary artery strips where H_2O_2 relaxes the cells precontracted by $PGF_{2\alpha}$ and hyperpolarizes the membrane potential [30]. In the cultured smooth muscle cells also phenylephrine induced a hyperpolarization which was almost completely inhibited by the α_1-receptor antagonist prazosin. Thus, it seems reasonable to conclude that the effect of phenylephrine in the cell preparation used

is mediated by α_1-receptors. It is known that the stimulation of α_1-receptors leads to the formation of inositol-1,4,5-trisphosphate (IP_3) via the phospholipase C pathway, and thereby to the release of Ca^{2+} from intracellular stores [31]. Moreover, it has been shown that α_1-receptor activation in smooth muscle cells lead to stimulation of a small cationic current and Ca^{2+}-dependent K^+ and Cl^- currents [32-36]. In freshly prepared muscle cells a depolarization of the cell membrane is observed following α_1-receptor activation. Whether the activation of voltage-dependent Ca^{2+} channels contributes to or follows the depolarization is still in debate [33,37-39]. Obviously, there is a difference in the prevalence or activity of channels stimulated or in stimulus transduction coupling between freshly prepared or short-term cultured and long-term cultured muscle cells.

In conclusion, the data presented in this paper suggest that the membrane hyperpolarization induced by H_2O_2 in cultured vascular smooth muscle cells is caused by an increase in $[Ca^{2+}]_i$ which in turn stimulates Ca^{2+}-activated K^+ channels.

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