A method of patch clamp recording in hyperbaric oxygen

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A method of patch clamp recording in hyperbaric oxygen is described which is derived from a method for patch clamping at high hydrostatic pressure. Excised patches equilibrate with hyperbaric oxygen within 10 minutes. Experiments with BK channels expressed in HEK 293 cells showed that 1 MPa O$_2$ caused an increase in the ionic permeability of the patch, an effect accelerated in the presence of ferrous ions. Seal resistance was unaffected. Small negative currents, (V$_{H}$ – 40 to – 65 mV, symmetrical KCl solutions), apparent in skewed all-points amplitude histograms, reduced the signal to noise ratio. In some patches the BK channel activity was inhibited and in others it persisted with the currents becoming irresolvable in the increased noise.

Oxygen toxicity, free radicals, BK channel, patch clamp

INTRODUCTION

A Method for Patch Clamp Recording in Hyperbaric Oxygen

It is well known that hyperbaric oxygen is toxic and a partial pressure of approximately 0.3 MPa or more causes convulsions in mammals (1 - 5). On the other hand hyperbaric oxygen therapy, in which carefully controlled doses of oxygen are administered, is successfully used in a number of conditions (6 -8). It is generally thought that the toxicity of hyperbaric oxygen arises from free radicals, which overwhelm the endogenous antioxidant defenses of organisms (9 - 11). Relatively few experiments have been carried out to study the electrophysiological effects of hyperbaric oxygen and thus far such studies have comprised classical recordings using large hyperbaric chambers (12). The main purpose of this report is to describe a method of carrying out patch clamp experiments in hyperbaric oxygen, which should extend and deepen our knowledge of some of the electrophysiological changes underlying oxygen toxicity and convulsions.

The preparation selected for this development work is the human brain BK channel expressed in HEK 293 cells. The channel’s large conductance makes it convenient for the purpose and often HEK 293 cells provide robust patches. It is conceivable that the channel
might be important in oxygen toxicity in humans, but the present report is primarily concerned with demonstrating the effectiveness of the hyperbaric oxygen patch clamp method.

METHODS

Cells and recordings

HEK 293 cells, expressing both α and β sub-units of the BK channel from human brain were obtained from Professor M. Ashford and colleagues, and were characterized by them. The cells were of the same line as in (13), and in the present work were used before confluence was achieved. All the experiments were carried out at 23 ± 2°C on inside-out patches, unless stated otherwise, using symmetrical Ca++-free recording solutions of composition, in mM, KCl 144, MgCl2 1, EGTA 10, Hepes 10 at pH 7.2. In some experiments ferrous ions (10uM ferrous ammonium sulphate) were added to the recording solution. Unpolished borosilicate glass patch pipettes, with a resistance of 5 M ohm in recording solution, were used in conjunction with an Axopatch 200A amplifier, a digital recorder (DTR-1204, Bio-Logic) and an A/D converter (CED 1401 Plus, Cambridge Electronic Instruments). Recordings were analyzed off line using Dempster software (14). The final signal was low pass filtered at an aggregate of 1KHz, with a sampling frequency of 10 kHz. The patches contained numerous channels so a holding potential, \( V_H \), was selected to elicit a low level of channel activity from an inside-out patch and recordings of 15-25 secs duration were made at suitable time intervals (5 – 15 min) throughout the experiment. Frequent checks were also made of the seal resistance using the 5mV test pulse built in to the patch clamp amplifier.

Hyperbaric apparatus

An apparatus designed for patch clamp recording at high hydrostatic pressure (15) was adapted for use with oxygen. In Fig 1A the patch pipette is seen fitted to an electrical connection that passes through the plug of a small steel pressure vessel. The tip of the patch pipette is immersed in recording solution, in a giant petri dish, which holds a cover slip supporting cultured cells. The legend to Fig 1 provides more details and explains how an excised patch is transferred to the pressure vessel with the tip of the patch pipette 0.9mm below the surface of the recording solution in the transfer bath. Equilibration with the applied partial pressure of oxygen takes place in 10 minutes. This figure is derived from mock-up experiments in which an oxygen electrode was mounted with its cathode 0.9mm below the surface of water and enclosed in a gas-proof chamber. Purging the chamber to change the pO_2 of the gas phase above the aqueous solution showed that the subsequent equilibration of oxygen in the solution in contact with the cathode took 10 min, in the absence of macroscopic convection. A calculated equilibration time for the diffusion of oxygen (D approximately 10^-5 cm^2 sec^-1) produced a similar result (16,17). In practice, convection doubtless arises in the recording bath in the pressure vessel from heat generated when the pO_2 is raised above ambient pressure, (see below). Convection arising from thermal effects and occasional vibrations will tend to accelerate equilibration. In the experiments reported here the equilibration time need not be known with precision, and the 10-min equilibration period is arbitrarily used in all cases to define the start of the exposure of the excised patch to the given partial pressure of oxygen. The temperature of the gas within the pressure vessel increased as the pO_2 was raised. Separate tests showed that compression to 0.3Mpa in one minute increased the temperature in the middle of the pressure vessel 2°C, and this heat dissipated in two minutes. The experiments used a compression of 0.3 MPa a minute to
1.0 MPa. This would have caused a transient rise in the gas temperature of much less than 6°C (as the conductivity of gas increases with density). The tip of the patch pipette, submerged 0.9 mm in 0.3 ml of recording solution would therefore have experienced a trivial rise and fall in temperature. The relatively high \( pO_2 \) of 1 MPa, was selected to maximize any effect of oxygen in the relatively short life time of the patches. This \( pO_2 \) is twice that to which mammals have been subjected in toxicity experiments (10,18,19), and the same as that used in a study of a Na, K-ATP-ase in rat brain slices (20).

**Figure 1**

[Diagram of patch clamp apparatus]

**Fig 1 -** Patch clamp apparatus for use with hyperbaric oxygen or other gases. (A) Pressure vessel plug [1] and its retaining ring [2], which are held in a modified Leitz micromanipulator (not shown). The electrical connection [3] through the plug is insulated by a teflon cone [4], which terminates in a socket [5] on the outer face of the plug, and on the inner face, it terminates as a cylindrical block [6], which supports the pipette holder [7] and the sliding support [8] for the transfer recording bath [9]. The patch-clamp amplifier headstage (not shown) connects to the socket [5]. The bath electrode [10] connection passes through the teflon block [6] to the vessel plug. The apparatus is shown with the tip of the patch pipette in a giant petri dish [11] (63mm int.diam. x 17mm deep) containing a reference electrode and cells grown on cover slips, not shown. The dish is mounted on the substage of an inverted microscope. After an excised patch is formed in the usual way, the level of the bath solution is raised to the dashed line, the pipette is also raised, and the transfer bath [9] is slid down and rotated under the tip of the pipette to provide a 0.3 ml recording bath, now served by its own bath electrode providing continuity of the recording circuit. The whole assembly is lifted clear and transferred to a stand to enable the level of solution in the transfer bath to be viewed through a binocular microscope and adjusted so the pipette tip is 0.9 mm below the surface, as measured against grid lines. The apparatus is then inserted into the pressure vessel, the internal diameter of which is 29 mm. (B). Initially air fills the vessel at normal atmospheric pressure, 0.1 MPa, to which oxygen contributes approximately 0.02MPa. Medical grade oxygen is bled into the vessel through the capillary [12] passing through the bottom plug [13], and equilibrates with the pipette holder through [14]. The pressure is measured using a Bourdon
gauge. The rate of compression used was 0.3 MPa min\(^{-1}\). The heat of compression was measured with a thermistor soldered to the teflon insulated connector [3]. The final pO\(_2\) in the experiments was 1 MPa additional to the pO\(_2\) of normal air and ignores water vapour pressure. The patch equilibrates with the hyperbaric oxygen in 10 min, (see Methods). This apparatus is a modified version of that described in (15) originally constructed for use at high hydrostatic pressure (up to 100 MPa, gas free).

RESULTS

Control experiments.

The experiments took the form of a pre-treatment period, when channel activity at the selected V\(_H\) was recorded at 5-15 min intervals, followed by a treatment phase. Experiments often lasted an hour or more. Many control experiments in which there was no treatment phase were therefore carried out at room pressure over a comparable time and these showed that the probability that the patch had an open channel varied a great deal but never declined to zero. Mode shifts were not seen. Single channel conductance, typically ~300 pS, determined from the unit current peak in an all-points amplitude histogram (14), was constant in control experiments. The signal to noise ratio, apparent in the histograms as the separation of the BK current peak from the baseline noise peak, also remained constant for long periods. When, in some control experiments the ratio became reduced, the seal resistance was also reduced, as judged from 5mV test pulses. It is important to stress that the amplitude of the BK current peak in the amplitude histograms reflects the channel activity (Po) and has no bearing on the signal to noise ratio.

Hyperbaric Oxygen

In the first set of experiments 1 MPa O\(_2\) was applied to an inside-out patch after an initial pre-treatment control period of about 20 minutes when the pO\(_2\) of the recording solution was normal, (ie pO\(_2\) approximately 0.02 MPa in normal air). In eleven out of eleven experiments there was a characteristic increase in the baseline noise in hyperbaric oxygen, clearly seen as small negative currents in the recordings and apparent as a broadening of the noise peak in all-points amplitude histograms. In more detail, there were two types of result. In one, exemplified in Fig 2a – c, the amplitude histogram shows separate peaks, corresponding to BK channel currents and noise, up to a certain stage. The peak attributable to BK currents then disappeared and the noise peak broadened (Fig 2c). This was seen in six of the eleven experiments. In the second type of result, seen in 5 of the 11 experiments, the BK currents persisted, becoming merged with a skewed, broadened noise peak (Fig 2d,e). The distinctive BK current pulses may be seen against a noisy background, i.e. the signal to noise ratio is reduced. In all cases seal resistance was unaffected. The time of the appearance of such skewed noise peaks in amplitude histograms was very variable (mean 17.8 ± SD 17mins, n=11) after equilibration with 1 MPa oxygen.

A separate set of six experiments was carried out in which ferrous ions (10µM) were present in the recording bath (in four cases before seal formation and in two cases after the inside out patch was excised). In five experiments the characteristic broadening of the noise peak was apparent by the time the 1 MPa O\(_2\) had equilibrated with the patch, i.e. within 10 minutes of applying oxygen pressure. The other, sixth, experiment showed the broadened noise peak 30 minutes after exposure with, as above, no change in seal resistance. The presence of ferrous ions (10 µM) at a normal pO\(_2\) had no effect on the signal to noise ratio (3 experiments), so clearly ferrous ions in the presence of 1 MPa oxygen accelerated the increase in noise, ie the deterioration of signal to noise ratio.
DISCUSSION

The effect of 1 MPa oxygen on the inside-out patch is to increase baseline noise through the appearance of small irregular negative currents, i.e. to decrease the signal to noise ratio. The skewed distribution seen in the amplitude histograms was sometimes accompanied by a loss of BK channel currents, (as in Fig 2a-c), but in other cases the BK currents were discernible as individual current pulses in recordings and apparent in skewed amplitude histograms. (Fig 2 d-e). To some extent this may reflect the number of BK channels present in the patch (as in Fig 2) but the data are insufficient to establish this generally. The presence of ferrous ions in the recording solution accelerated the effect of hyperbaric oxygen, which is consistent with free radicals being involved.

Oxygen is known to generate superoxide radicals (O$_2^-$), hydroperoxyl radicals (H00.) and singlet oxygen (1O$_2$). Hydrogen peroxide formed from superoxide radicals yields, through the Fenton reaction (2,9,21), hydroxyl radicals (H0.), a proportion of which will be scavenged by HEPES (22). As oxygen partitions in hydrophobic phases some 5-8 times more than in water, abundant radicals are likely to be generated at the pO$_2$ used here, in both aqueous and lipid phases. Radicals formed in bilayers will distribute themselves within it according to their charged nature. Hydroperoxyl radicals, for example, are more hydrophobic than superoxide anion radicals and will stay away from the bilayer-aqueous interface. Superoxide radicals, however, are highly reactive in a hydrophobic phase (22).

The patch clamp recordings reveal the onset of an increased ionic permeability in hyperbaric oxygen, which may be similar to that seen in other in preparations. For example, Labarca & Latore (23) observed spontaneous channel like conductance changes in planar bilayers made of oxidised lipids. The present authors have seen similar conductance fluctuations in patch-clamped liposome bilayers made of oxidized lipids. The conductance fluctuations varied; some were small, as here, and others were very large (500 pS) and both were abolished by the anti-oxidant 2,6-Di-tert-butyl-4--methylphenol, (in preparation). The permeability of liposomes subjected to lipid peroxidation is increased although the relationship between permeability and oxidation is not simple (24 - 26). However there are reasons for thinking that the ionic leakiness, apparent in the membrane patches as small negative currents (e.g. Fig 2), may not arise from lipid oxidation. The work of Deuticke (27) on the permeability of erythrocytes shows that membrane proteins are more susceptible to oxidation (through the reactivity of methionine and cysteine), than lipids (28). Furthermore hyperbaric oxygen (0.5 MPa) can cause extensive protein oxidation in rat brains, whilst lipid peroxidation is confined to specific regions (18). Guinea pig and cardiac cells manifest a non-specific cation conductance when activated by a free radical generating system (29,30).

The distinctive currents recorded in hyperbaric oxygen may be caused by free radical damage because the changes seen at a given $V_H$ are accelerated in the presence of ferrous ions, whilst the seal resistance at 5mV is unaffected. Hydrostatic pressure is not a factor here (15). Other patch clamp experiments involving free radical damage have similarly shown seal resistance to be unaffected (29,30). During the early stage in the deterioration of the ionic permeability barrier some of the BK channels remained able to function (Fig 2d, e). With the loss of an adequate signal to noise ratio it becomes impossible to detect their currents, so how long the BK channels’ function remains unaffected is unknown.

In other patches BK channels ceased to function, or their $P_0$ was greatly reduced, somewhat earlier in the progressive increase in noise (in 6 of 11 iron-free experiments, including
The BK channel structure itself may be affected directly by free radicals or, indirectly, by the products of reactions in the surrounding membrane (31).

Figure 2

Fig 2 - Recording of BK channel currents in an inside-out patch from HEK 293 cells subjected to a pO$_2$ of 1 MPa. Recordings were made at time intervals after mounting the patch in the pressure vessel at time zero (V$_{H}$ = -40mV). All-points histograms are shown (vertical axis, percent of total number of sample points; horizontal axis, current bins) with, beneath, a short corresponding section of recording: (a), at 10 mins, the patch is equilibrated with 0.02 MPa O$_2$, in normal air. Note the signal to noise ratio is given by the separation of the BK channel current peak (-13.5pA) from the noise peak (0pA); (b), at 17 mins, one minute after a pO$_2$ of 1 MPa had been applied. The reduced amplitude of the BK channel peak in (b) is unimportant as such variation is often seen in control experiments; (c), at 42 min and 16 min after equilibration with oxygen, small negative currents have appeared. These are apparent in the amplitude histogram as a broadened noise peak. The BK channel currents have disappeared; (d) & (e), are recordings from another experiment in which the BK channel currents are discernible after the baseline shows small negative currents. In (d), V$_{H}$ = 40mV, the patch has been equilibrated with 1 MPa O$_2$ for 2 min and the signal to noise ratio is normal. Unit current is –11 pA in this experiment. In (e), the patch has been equilibrated for 12 min.
and the BK channel current peak has become merged with the broadened noise peak. The signal to noise ratio is reduced. In both experiments the seal resistance was unaltered.

The method described in this paper should enable a variety of cell membranes and ion channels to be subjected to hyperbaric oxygen and studied by the patch clamp technique. The advantages of the latter are well known but the lifetime of the patch may prove a significant limitation.

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REFERENCES