Biochimica et Biophysica Acta, 1147 (1993) 165–167

BBAMEM 70719

Rapid Report

The voltage-gated non-selective cation channel from human red cells is sensitive to acetylcholine

P. Bennekou

August Krogh Institute, University of Copenhagen, Copenhagen (Denmark)

(Received 2 February 1993)

Key words: Erythrocyte; Cation channel; Acetylcholine receptor; Carbachol; Nicotine; Patch clamp

Using patch-clamp technique it is demonstrated, that the voltage-gated non-selective cation channel present in the human red cell is coupled to an acetylcholine receptor of nicotinic type. The concentration dependence of carbachol, the potency of selected agonists and an estimate of the numbers of channels/red cell are reported.

The K^+ and Na^+ conductances of the human red cell, the ground leaks, are under physiological conditions orders of magnitude lower than the corresponding conductances in most other cell types, and orders of magnitude lower than the red cell anion conductance [1]. However, as part of a pump/leak system [2] these cation conductances are crucial for the long term maintainance of the low red cell membrane potential (−12 mV) [3], which with physiological ion gradients must be based on leak pathways for K^+ and Na^+ having a conductance ratio close to 1. Recently, the presence in the human red cell membrane of a voltage-dependent non-selective cation channel with equal conductances for K^+ and Na^+ was demonstrated in single channel experiments [4].

Further experiments have shown, that this channel is activated by the acetylcholine receptor agonists acetylcholine, carbachol and nicotine and consequently can be characterized as an acetylcholine receptor channel of nicotinic type. It is believed, that the original observation of this channel [4] under nominally agonist-free conditions, can be explained by a contamination of utensils with nicotine due to tobacco smoking which activated the channel.

Red cells from freshly tapped blood from healthy, non-smoking human donors were washed in approx. 30 vol. ice-cold isotonic KCl and stored on ice. Small aliquots of the cell suspension were transferred to the experimental chamber and diluted to a final cytocrit of approx. 0.005% in a solution containing 500 mM KCl, 5 mM Mops, 22 μM $CaCl_2$, 1 mM EGTA, adjusted to pH 7.4, by titration with NMGA (3–4 mM). The pipette (soft glass, resistance in 150 mM salt solution 10 MΩ) was filled with an identical solution, to which was added the agonist in varied concentration. After giga-seal formation, inside-out patches were formed moving the pipette tip with the attached cell through the Ringer/air interface. All experiments were performed at room temperature. In order to activate the channel [4], the pipette potential was kept at −100 mV, corresponding to a membrane potential of +100 mV in the inside-out configuration. Since the length of individual open and closed events are in the millisecond to minute range, individual experiments have typically been performed with durations from 20 to 45 min in order to get representative runs.

The signal from the patch clamp amplifier (EPC-7, List Electronic) was filtered (3 kHz), digitized (44100 samples/s, 16 bit datasize) and stored on videotape. For analysis the digitized data were transferred to a computer and further filtered digitally, using a Gauss filter with a cut off frequency of 500 Hz.

The single-channel open state probability, p_o, was estimated from a fit of the current histogram, see Fig. 1, to a sum of Gaussians. Assuming the channels to be identical and independent, the relative area under the zero current peak, calculated from the parameters to the fit, was taken to be the nth power of the single channel closed state probability, p_c, where n represents the number of channels present in the patch. The single-channel open state probability is then found as $p_o = 1 - p_c$.

Correspondence to: P. Bennekou, August Krogh Institute, University of Copenhagen, Universitetsparken 13, 2100 Copenhagen Ø, Denmark.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*,-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; NMGA, *N*-methyl-D-glucamine.

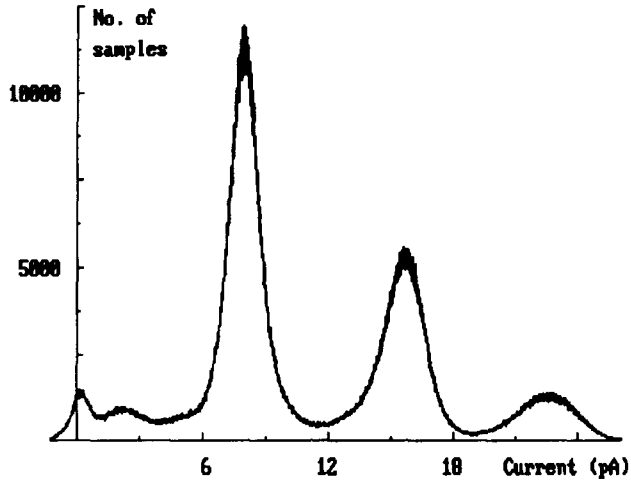

Fig. 1. Current distribution for an experiment with 2.5 μM carbachol in the pipette. The peak at zero pA represents the well defined outcome: all channels closed. The parameters from a fit to a sum, in this case 5, of Gaussians was used to calculate the relative area under the zero-current peak. Note that due to the occurrence of substates the number of open channels under the individual peaks are not constant. Consequently, the distribution of current levels can not be fitted to a binomial distribution.

Experiments performed with the cholinergic agonists carbachol, see Fig. 2, nicotine and acetylcholine show an increase in the duration and frequency of channel openings with increasing concentrations until a maximum in p_o is reached. With a further increase of agonist concentration relatively long inactive periods is observed, resulting in a decline in the single channel

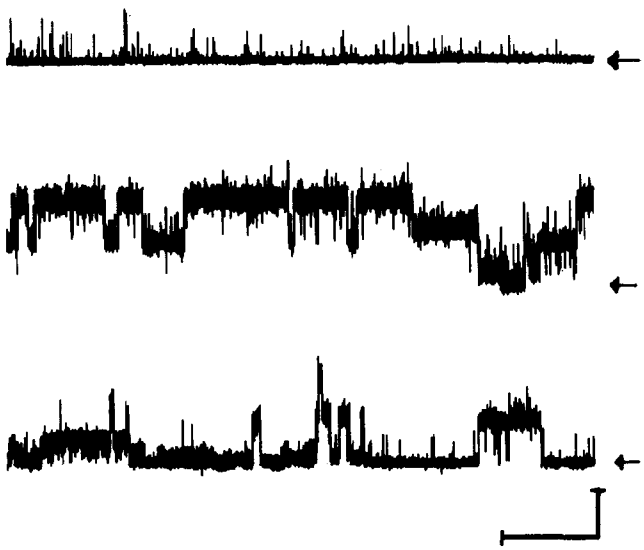

Fig. 2. Current traces from experiment with varied carbachol concentration in the pipette. Top: 1 μM; middle: 2.5 μM and bottom: 100 μM. The zero current levels (all channels closed) are indicated by arrows. In the experiment shown in the top trace 1 channel was present, while 3 were present in the experiments shown in the middle and bottom traces. Note that substates can be seen in the two lower traces. The horizontal bar (bottom) represents the time scale, 10 seconds, the vertical bar the current scale, 10 pA.

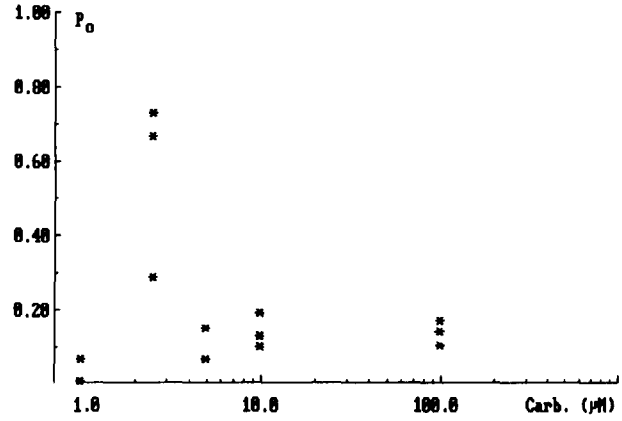

Fig. 3. Single-channel open state probability from individual experiments shown as function of the carbachol concentration.

open state probability. The maximum activity is found to occur at 2.5 μM carbachol, see Fig. 3, close to 1 μM acetylcholine and still lower for nicotine, which is the most potent agonist, showing stimulation from 10^{-10} M (not shown).

The absence of channels in a patch forming a gigaseal was infrequent, and more than one channel/patch was frequently observed, see Table 1, with 4 as the maximum in the present series of experiments. However, estimation of the number of channels in the patch is not straightforward, since the open channel can occupy a number of states with conductance levels both below and above the most frequent state [5]. At least some of these states, including the high conductance ones (superstates) are close to multiples of some unit conductance. Thus two or more simultaneously open channels occupying substates can be difficult or impossible to discern from a single channel which has opened to the main state or a superstate, thus sharing the same current level. The number of channels in the patch was consequently estimated from a visual inspection of the current traces as the maximum number of concurrent openings, which has been shown [6] to be the optimal method when the number of channels is $\leqslant 4$. Even with the long runs used, it is possible that the number of channels is underestimated in experiments where the activity is low, since simultaneous openings become very rare. However, the mean num-

TABLE I

Carbachol (μM)	Number of expts.	Channel/patch (mean)	p_o mean ± S.D.
1	6	1.5	0.022 ± 0.030
2.5	3	2.0	0.559 ± 0.239
5	2	1.0	0.106 ± 0.057
10	3	2.3	0.115 ± 0.019
100	4	2.0	0.120 ± 0.046
All	18	1.8	

ber of channels in the patches is fairly constant over the carbachol concentration range, see Table I, which argues against an significant error in the estimate. Furthermore, an underestimate at low agonist concentration, would tend to underrate the apparent agonist activation, not emphasize it.

The number of channels in the red cell membrane can be estimated at ≈ 300 channels per red cell using the mean number of channels per patch of 1.8, see Table I, an estimate of the patch area at 1 μm^2 of and 10 MΩ pipette [7] and the red cell area of 1–60 μm^2.

The presence of an acetylcholine-activated non-selective cation channel in the human red cell is in accordance with reports on macroscopic systems. In a study of the effect of neurotransmitters on red cell membrane rigidity, using spin-labeling [8], it was found, that the membrane rigidity, expressed as the order parameter of an induced nitroxide group, and cation permeability increased as function of the carbachol concentration, with a maximum at 1 μM. It was found, that atropine and TTX blocked this effect, but the latter only in the presence of a Ca^{2+} chelating agent, whereas curare had a less pronounced inhibitory effect.

Tang et al. [9] found that carbachol (100 μM) transiently stimulated Ca-uptake in human red cells followed by an increase in the level of cyclic GMP, an effect that was blocked by atropine (100 μM). This stimulation of cGMP was also observed following addition of the Ca^{2+} ionophore A23187, but not seen in the presence of calcium chelating agents (EGTA).

In the above mentioned reports [8,9], it was concluded, that the experiments indicated the presence of a cholinergic receptor of muscarinic type, since the effects were blocked by the muscarinic antagonist atropine, and since 23 fmoles/mg membrane protein muscarinic receptors have been reported to be present in the human red cell membrane [10]. This corresponds to about 10 receptors red cell, using a value of 8000 mg membrane protein per litre packed red cells [11], a number that is so low that the presence of a muscarinic receptor in a patch should be a rare event. Furthermore, it would be expected that muscarinic receptors would be associated with potassium-channel activity regulated by a G-protein and second messenger chain [12] and not a non-selective cation channel. It should be noted too, that the channel is functioning for hours in an excised patch in the inside-out configuration, which makes it improbable, that the second messenger system associated with a muscarinic receptor is intact.

Regarding the blocking effect of atropine [8,9], this effect is not necessarily specific for the muscarinic receptor. It has been reported [13] that nicotinic acetylcholine channels in bullfrog sympathetic ganglion cells are blocked by this compound. Since the nonselective cation channel in human red cells is permeable to Ca^{2+} (unpublished result) the stimulation of cGMP could as well be triggered by a calcium influx through this channel as through a activation of a transport pathway associated with a muscarinic receptor. Furthermore, the changes in permeability for monovalent cations reported in Refs. 8 and 9 points more to a nicotinic type of non-selective channel than to an effect due to a muscarinic receptor.

The channel, the basic characteristics of which are described above and in Ref. 4 have many properties in common with the established set of nicotinic acetylcholine channels, among these the lack of selectivity to monovalent cations, calcium permeability, conductance level, display of substates, and apparent desensitization (long silent periods) at high agonist concentrations. Regarding the voltage dependence, however, the situation seems to be different, since the present channel is activated at positive membrane potentials. The significance of this is not known at present, nor is the physiological function in the red cell of an acetylcholine-activated channel established. Both these questions is presently under investigation. It should be noted however, that it has been shown, that at least some acetylcholine channels can open spontaneously in the absence of agonist, but with a very low open state probability [14], an observation that accords well with the steady state behaviour of the human red cell.

This project was supported by the Carlsberg Foundation (88-0036) and the NOVO Foundation (198812-12-1).

References

1 Hunter, M.J. (1971) J. Physiol. 218, 49P–50P.
2 Tosteson, D.C. and Hoffman, J.F. (1960) J. Gen. Physiol. 44, 169–194.
3 Hladky, S.B. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lev, V.L., eds.), pp. 173–175. Academic Press, London.
4 Christophersen, P. and Bennekou, P. (1991) Biochim. Biophys. Acta 1065, 103–106.
5 Bennekou, P. and Christophersen, P. (1991) Acta Physiol. Scand. 143, 1 (A20).
6 Horn, R. (1991) Biophys. J. 60, 433–439.
7 Sakmann, B. and Neher, E. (1983) in Single channel recording (Sakmann, B. and Neher, E., eds.), pp. 37–52, Plenum Press, New York.
8 Huestis, W.H. and McConnell, H.M. (1974) Biochem. Biophys. Res. Commun. 57, 726–733.
9 Tang. L.C., Schoomaker, E. and Weismann, W.P. (1984) Biochim. Biophys. Acta 772, 235–238.
10 Aromstam, R.S., Abood, L.G. and MacNeil, M.K. (1977) Life Sci. 20, 1175–1180.
11 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 105–115.
12 Receptor nomenclature Supplement (1992) Trends Pharmacol. Sci. Suppl. January.
13 Minota, S., Eguchi, T. and Kuba, K. (1989) Pflügers Arch. 414, 249–256.
14 Jackson, M.B. (1984) Proc. Natl. Acad. Sci. USA 81, 3901–3904.