Diphtheria toxin at low pH depolarizes the membrane, increases the membrane conductance and induces a new type of ion channel in Vero cells

Stig Eriksen, Sjur Olsnes¹, Kirsten Sandvig¹ and Olav Sand²

Department of Biology, University of Oslo, PO Box 1051, Blindern, N-0316 Oslo and ¹Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway

²Corresponding author

Communicated by S.Olsnes

Receptor-dependent translocation of diphtheria toxin across the surface membrane of Vero cells was studied using patch clamp techniques. Translocation was induced by exposing cells with surface-bound toxin to low pH. Whole cell current and voltage clamp recordings showed that toxin translocation was associated with membrane depolarization and increased membrane conductance. The conductance increase was voltage independent, with a reversal potential of ~15 mV. This value was unaffected by changing the Cl⁻ gradient across the membrane and microfluorometric measurements showed that the cytosolic Ca²⁺ concentration was only marginally elevated by the translocation. The conductance increase is thus mainly due to monovalent cations. Exposing outside-out and cell-attached patches with bound toxin to low pH induced a new type of ion channel in the membrane. The channel current was inward at negative membrane potentials and the single channel conductance was ~30 pS. This value is about three times larger than for receptor-independent channels induced by diphtheria toxin or toxin fragments in artificial lipid membranes.
Key words: diphtheria toxin/ion channels/membrane translocation/patch clamp recordings/Vero cells

Introduction

In recent years there has been considerable focusing on bacterial protein toxins with intracellular targets. Tetanus and botulinum toxins act by proteolytic cleavage of proteins required for exocytosis in neurons and other cells (Schiavo *et al.*, 1992; Südhof *et al.*, 1993). Cholera and pertussis toxins act by ADP-ribosylating α -subunits of trimeric G-proteins, thus interfering with transmembrane signal transduction; *Shigella* toxin and a number of plant toxins inactivate ribosomes; *Pseudomonas* exotoxin A and diphtheria toxin inactivate elongation factor 2 and thus inhibit protein synthesis. Anthrax toxins, *Clostridium difficile* toxin, *Clostridium botulinum* C3 toxin and others also act on intracellular targets (for review, see Olsnes *et al.*, 1991). Common to all these toxins is that they translocate an enzymatically active polypeptide across cellular membranes into the cytosol.

Membrane translocation of proteins is an important

process in all cells. In bacteria, proteins are secreted from the cytosol across the bacterial membrane, whereas in eukaryotes proteins are translocated from the cytosol into several types of organelles. Export proteins are translocated into the endoplasmic reticulum followed by exocytosis (Rapoport, 1992). Other proteins are translocated into peroxisomes, chloroplasts and mitochondria (Glick *et al.*, 1992). These processes are now understood in considerable detail and it appears that ion-conducting channels are formed in membranes where translocation takes place (Simon and Blobel, 1991, 1992).

Among toxins with intracellular sites of action, diphtheria toxin has been most extensively studied with respect to membrane translocation. An important reason for this is that diphtheria toxin can be induced to translocate directly across the plasma membrane when cells with surface-bound toxin are exposed to low pH (Moskaug *et al.*, 1988). The toxin is normally taken up by endocytosis (Morris *et al.*, 1985) and the subsequent translocation across the endosomal membrane is induced by acidification of the endosome (Draper and Simon, 1980; Sandvig and Olsnes, 1980). The toxin is a water soluble polypeptide with a region particularly sensitive to proteolytic cleavage. Such cleavage (nicking), which results in an A- and a B-fragment linked together by a disulfide bond, is a requirement for cell toxicity (Collier and Kandel, 1971). The B-fragment binds to cell surface receptors, whereas the A-fragment is translocated into the cytosol and inhibits protein synthesis by ADP-ribosylating elongation factor 2 (Pappenheimer, 1977). Papini *et al.* (1988) and Sandvig and Olsnes (1988) have demonstrated the induction of fluxes of monovalent cations across the surface membrane of Vero cells pretreated with diphtheria toxin and then exposed to low pH. Similar findings regarding anthrax toxin were recently reported by Milne and Collier (1993).

Kagan *et al.* (1981) showed that a deletion mutant of diphtheria toxin, lacking the receptor-binding part of the B-fragment, induces ion channels in artificial lipid membranes. The property of channel induction was localized to the B-fragment and the single channel conductance observed was ~10 pS. In similar experiments, Donovan *et al.* (1981) showed that normal diphtheria toxin also has channel forming properties in lipid bilayers. The channel formation of both intact toxin and the B-fragment have since been studied in considerable detail in artificial membranes (Misler, 1983, 1984; Zalman and Wisnieski, 1984; Shiver and Donovan, 1987). However, in all these studies channel formation was induced in artificial systems lacking diphtheria toxin receptors, whereas translocation *in vivo* was found to be strictly dependent on the specific toxin receptor (Stenmark *et al.*, 1988), which is identical to the uncleaved precursor of heparin-binding epidermal growth factor (Naglich *et al.*, 1992). The biological significance of the channels formed in artificial mem-

branes, with respect to *in vivo* toxin translocation into cells, is thus uncertain. Tetanus toxin (Boquet and Duflo, 1982; Borochov-Neori *et al.*, 1984), botulinum toxin (Donovan and Middlebrook, 1986) and anthrax toxin (Blaustein *et al.*, 1989) were also found to form channels in artificial membranes. In the present work we have studied the effect of low pH on cells exposed to diphtheria toxin using patch clamp techniques and we demonstrate that at low pH the toxin induces a new type of ion channel in Vero cells.

Results

Electrical membrane properties of Vero cells

The normal electrical membrane properties of Vero cells were studied using the whole cell patch clamp method in current clamp mode. The input resistance of single, independent cells was $4.0 \pm 1.6 \text{ G}\Omega$ (mean $\pm$ SD, $n = 19$), whereas the input resistance of clustered cells varied between 50 and 500 M Ω . The input resistance was highest for cells peripherally positioned in the cluster. The membrane potential showed no corresponding variation and was $-32.7 \pm 9 \text{ mV}$ ($n = 68$) in single cells and $-33.3 \pm 6.1 \text{ mV}$ ($n = 18$) in clustered cells. These findings are in agreement with the existence of gap junctions between neighbouring Vero cells. In the following, only single, independent cells were used to study the toxin effects using the whole cell patch clamp method.

Effects of diphtheria toxin at low pH on the membrane potential and input resistance of Vero cells recorded during current clamp

In the initial investigation of toxin effects the whole cell method was employed in current clamp mode and sample recordings in different solutions were compared. Recordings at pH 7.4 were obtained within 45 min after addition of 1 $\mu\text{g/ml}$ nicked toxin to the normal recording buffer. This solution was then replaced with a corresponding solution at pH 4.5 and the recordings continued for $\sim 1 \text{ h}$. The mean membrane potential and input resistance before the pH reduction were $-32.0 \pm 6.8 \text{ mV}$ ($n = 30$) and $4.5 \pm 1.5 \text{ G}\Omega$ ($n = 30$) respectively. The pH decrease caused a depolarization to $-10.2 \pm 5.7 \text{ mV}$ ($n = 23$) and a reduction of the input resistance to $1.3 \pm 0.6 \text{ G}\Omega$ ($n = 23$). In control experiments without diphtheria toxin the mean membrane potential and input resistance were $-31.8 \pm 6.5 \text{ mV}$ ($n = 13$) and $4.3 \pm 1.4 \text{ G}\Omega$ ($n = 13$) in the normal recording solution and $-31.5 \pm 6.3 \text{ mV}$ ($n = 13$) and $4.4 \pm 1.2 \text{ G}\Omega$ ($n = 13$) after the pH reduction. These latter values are not significantly different from the values obtained before the pH reduction (paired *t*-test, $P < 0.05$).

These initial sample recordings clearly showed that diphtheria toxin at low pH causes membrane depolarization and reduced input resistance in Vero cells. To study the time dependence and reversal potential of this response, the effects of pH reduction on toxin-exposed cells were studied during continuous recordings from single cells. To allow time for maximal binding of the toxin to its membrane receptors, 1 $\mu\text{g/ml}$ nicked diphtheria toxin was added to the recording medium 30 min prior to the recording session. The effect of low pH was then tested by ejection of the low pH solution directly on to the

Fig. 1. Whole cell current clamp recordings of the membrane response (middle trace) evoked by exposing Vero cells with surface-bound diphtheria toxin to low pH. The input resistance was monitored by injecting hyperpolarizing current pulses (lower trace) at regular intervals. Low pH (4.5) solution was pressure ejected (upper trace) through a micropipette positioned close to the cell. The cells were preincubated for 30 min at pH 7.4 in solution containing 1 $\mu\text{g/ml}$ nicked toxin. Lowering the pH depolarized the cell and reduced the input resistance.

recorded cells. Figure 1 displays the result of one of these experiments. In this whole cell current clamp recording the input resistance was monitored by injecting hyperpolarizing current pulses at regular intervals. The pH reduction caused an immediate depolarization and reduced input resistance. The delay between the start of the ejection and the response varied between <1 and 2 s. In this series of experiments, 44 cells were tested and 40 of these showed a response similar to the one illustrated in Figure 1. The remaining four cells showed no response. In control experiments, ejecting low pH solution onto Vero cells in toxin-free, normal solution did not induce changes in membrane potential or input resistance ($n = 24$).

To test if the observed effects were non-specific or dependent upon the existence of membrane receptors for diphtheria toxin, we also performed similar experiments on rat pituitary GH₄ cells, which lack such receptors. Ejection of low pH solution onto toxin-exposed GH₄ cells did not evoke changes in membrane potential or input resistance ($n = 9$).

Effects of diphtheria toxin at low pH on the electrical membrane properties of Vero cells recorded during voltage clamp

To study further the voltage dependence of the diphtheria toxin-induced conductance increase and its reversal potential, we employed the whole cell patch clamp method in the voltage clamp mode. The holding potential was -57 mV and the 300 ms commands varied in 20 mV steps from -97 to 23 mV . The duration between steps was 1 s. The experimental procedure was similar to that described for the current clamp experiments. The first set of commands was given just prior to ejection of the low pH solution and a second set was given just before terminating the ejection. Figure 2 shows the resulting current recordings in one of these experiments and Figure 3 gives the corresponding *I/V* curves. At the holding potential the pH reduction induced a large inward current, corresponding to the depolarization observed in current clamp. The larger current steps recorded at low pH compared with the control values reflect the increased membrane conductance previously measured in current clamp. The *I/V* curves were linear both before the ejection and at the low pH, showing a voltage-independent membrane conductance at both pH values. The diphtheria toxin-induced conductance increase is therefore also voltage independent. The two *I/V* curves intersect at a membrane

Fig. 2. Whole cell voltage clamp recordings showing superimposed current traces recorded from a Vero cell with surface-bound diphtheria toxin before (A) and during (B) exposure to low pH. The holding potential was -57 mV and the commands varied in 20 mV steps from -97 to 23 mV. Lowering the pH induced a large conductance increase and a pronounced inward current at the holding potential.

Fig. 3. I/V curves from a Vero cell with surface-bound diphtheria toxin obtained before and during exposure to low pH. The curves are based on the recordings shown in Figure 2. The current was measured 200 ms after onset of the command pulses. Both I/V curves are linear. The curves intersect at -10 mV, which is the reversal potential of the response evoked by low pH.

potential of 10 mV. At this potential, which is the reversal potential of the diphtheria toxin-induced conductance increase, the ejection had no effect on the membrane current compared with the control value. Similar measurements were performed on nine additional cells and the mean reversal potential in all these experiments was 14.5 ± 14.7 mV. This value is distant from the equilibrium potential of any of the relevant ion species, indicating that the diphtheria toxin-induced conductance increase is relatively non-specific.

To study the specificity of the diphtheria toxin-induced conductance increase in more detail, additional voltage clamp experiments were performed in a solution where 80% of the Cl^- was replaced with gluconate. This was achieved by reducing the NaCl concentration to 18.1 mM and adding 131.9 mM Na-gluconate. This would shift the reversal potential by ~ 40 mV in the positive direction for a conductance increase solely to Cl^- . However, the observed reversal potential was unaffected by the altered concentration gradient for Cl^- across the membrane. In the 10 experiments with lowered Cl^- concentration the reversal potential was 11.3 ± 13.8 mV, indicating that

Fig. 4. Inward current responses to low pH exposure of Vero cells incubated in medium with varying concentrations of diphtheria toxin. The records were obtained during whole cell voltage clamp at a holding potential of -57 mV. The current response was measured 20 s subsequent to start of pressure ejection of the pH 4.5 solution. The responses are presented as a percentage of the mean value at a toxin concentration of 1 $\mu\text{g/ml}$. At this toxin concentration the response was also measured in the presence of 1 mM Cd^{2+} (open symbol). The data are presented as mean values $\pm$ SD and the number of recorded cells at each toxin concentration is indicated on the figure.

diphtheria toxin induces a conductance increase mainly to cations.

Figure 4 shows the dose-response relationship of the toxin-dependent inward current induced by lowering the pH. Different culture dishes were preincubated at toxin concentrations of from 0.01 to 3 $\mu\text{g/ml}$ and the maximum inward current induced by low pH was recorded at a holding potential of -57 mV from several cells within each dish. These experiments were time consuming and we were not able to obtain sufficient data at all the toxin concentrations within a single day. The figure therefore pools data recorded from different cell culture batches 1–3 days after seeding. The magnitude of the response showed some variation between the different cell culture

batches. To be able to compare the responses obtained on separate days, the inward current is given as a percentage of the mean response at a toxin concentration of 1 $\mu\text{g/ml}$, which was always included as a reference. The inward current response was clearly saturable, with maximal and half-maximal responses at $\sim 1 \mu\text{g/ml}$ and $0.1 \mu\text{g/ml}$ respectively. This is in good agreement with previously obtained binding data (Olsnes and Sandvig, 1986).

We have previously shown that 1 mM Cd^{2+} strongly inhibits both toxin-dependent Na^+ uptake and K^+ efflux in Vero cells (Sandvig and Olsnes, 1988). In the present study we therefore measured the effect of 1 mM Cd^{2+} on the inward current response at a toxin concentration of 1 $\mu\text{g/ml}$. The cell cultures were preincubated with both Cd^{2+} and the toxin and the inward current response induced by low pH was then reduced by $\sim 70\%$ (Figure 4).

Effects of diphtheria toxin at low pH on the cytosolic Ca^{2+} concentration of Vero cells

To investigate the effect of diphtheria toxin at low pH on the cytosolic Ca^{2+} concentration, we performed microfluorometric measurements on fura-2-loaded Vero cells. The cells were preincubated with 1 $\mu\text{g/ml}$ nicked toxin at pH 7.4 for 30 min and the effect of lowering the pH was then studied by pressure ejection of the pH 4.5 solution close to the recorded cells. In 16 of the 33 cells tested in these experiments, no elevation in cytosolic Ca^{2+} level was observed. The remaining cells showed a moderate increase of $<60 \text{ nM}$. In control experiments the cells were stimulated by pressure ejection of 10 μM ionomycin, which is a Ca^{2+} ionophore. This treatment promptly elevated the cytosolic Ca^{2+} concentration from $\sim 100 \text{ nM}$ to 700–800 nM. From these experiments we conclude that the diphtheria toxin-induced conductance increase is probably mainly due to monovalent cations.

Endogenous ion channels in Vero cells

The endogenous channel activity of Vero cells was studied in outside-out and cell-attached patches in recording solution without toxin. In all, $\sim 80\%$ of the patches showed channel activity, with only one channel type being evident under our recording conditions at both pH 7.4 and 4.5. An example of this channel in normal recording buffer is presented in Figure 5, showing channel openings in an outside-out patch at different holding potentials. The channel activity showed no voltage dependency. The corresponding I/V relationship for the single channel current is shown in Figure 6. The relationship was linear between -17 and -57 mV , with a slope conductance of 45 pS. The channel current reversed at -77 mV , which is close to the equilibrium potential for K^+ ($E_{\text{K}} = -85 \text{ mV}$). This endogenous channel is therefore most likely a K^+ channel. However, the ion selectivity and other properties of the channel were not studied further.

At low pH diphtheria toxin induces ion channels in the Vero cell membrane

To study the nature of the toxin-induced conductance increase in more detail, outside-out patches from Vero cells pretreated with 1 $\mu\text{g/ml}$ nicked diphtheria toxin were exposed to low pH solution. This treatment induced a new type of ion channel in four of the eight patches tested; a typical example of the channel activity at different potential

Fig. 5. Endogenous, single channel activity in an outside-out patch from a Vero cell in normal solution. The patch was voltage clamped at different holding potentials, as indicated on the figure. This channel is representative for the only endogenous channel type being evident in Vero cells under the present recording conditions at both pH 7.4 and 4.5.

Fig. 6. I/V relationship for the channel displayed in Figure 5. The channel current reversed at -77 mV and the slope conductance was 45 pS within the linear range between -17 and -57 mV .

levels is shown in Figure 7. The corresponding I/V relationship, which was linear with a slope conductance of 27 pS within the studied potential range, is given in Figure 8. At negative membrane potentials the channel current was inward through these channels, as opposed to the endogenous channels, where the current reversed close to -80 mV (Figure 5). Around this potential level it was only possible to observe current through the toxin-induced channels. In control experiments on cells not exposed to the toxin, low pH never induced this type of ion channel. The activity of the induced channels was voltage independ-

Fig. 7. A new type of single channel activity recorded in an outside-out patch from a Vero cell with surface-bound diphtheria toxin. The patch was voltage clamped at different holding potentials, as indicated on the figure. A channel with properties distinctly different from the endogenous type was activated immediately after exposing the patch to low pH. The current through this channel was inward at negative membrane potentials. At -57 mV, opening of endogenous channels was also detected in the same patch.

Fig. 8. I/V relationship for the channel displayed in Figure 7. The relationship was linear within the tested voltage range, with a single channel conductance of 27 pS.

ent and the mean single channel conductance was 28.1 ± 4.3 pS ($n = 4$). In the outside-out recording mode the induced channel activity was of short duration and it was difficult to obtain sufficient data to accurately estimate the reversal potential of the channel current. However, the reversal potential was evidently in the vicinity of 0 mV.

The making of an outside-out patch severely interferes with intracellular structures and with the solute composition on the cytosolic side of the patch. This may explain why only half of these patches showed induced, short lasting channel activity. We therefore also studied the effect of low pH on toxin-exposed cells using the cell-attached recording mode, which leaves the intracellular milieu intact. This was simply achieved by filling the electrodes with the pH 4.5 solution and recording from cells pre-exposed to 1 μ g/ml nicked toxin in the normal recording medium. In all of the 25 patches studied in this series of experiments, the effects of the low pH electrode

solution were evident as a dramatic conductance increase immediately after giga-seal formation. In only 30% of the cases was the initial conductance increase sufficiently moderate to resolve single channel activity. The mean single channel conductance of the toxin-induced channels in these cell-attached patches was estimated as 31.0 ± 15.3 pS ($n = 8$). The pooled data from both the outside-out and the cell-attached recording modes give a mean single channel conductance of 30.2 ± 11.9 pS ($n = 12$).

Discussion

The main finding in the present work is that receptor-bound toxin exposed to low pH induces the formation of cation-selective channels with a conductance of ~ 30 pS. Our electrophysiological observations are in agreement with previous biochemical studies. Toxin-induced depolarization of Vero cells has previously been observed by using a fluorescent probe (Papini *et al.*, 1988). Sandvig and Olsnes (1988) studied ion fluxes associated with the translocation of diphtheria toxin across the Vero cell membrane. They found that exposure of cells with surface-bound toxin to low pH, to induce toxin entry, increased the transmembrane fluxes of Na^+ , K^+ and H^+ , whereas the fluxes of Cl^- and uncharged molecules were unaffected. It was concluded that translocation of diphtheria toxin across the cell membrane increases the membrane fluxes of monovalent cations, probably due to channel formation. That conclusion is supported by the present whole cell recordings, showing a toxin-induced conductance increase with a reversal potential of 14.5 mV. The reversal potential was unaffected by changing the Cl^- gradient across the membrane and, also in agreement with the previous measurements (Sandvig and Olsnes, 1988), the cytosolic Ca^{2+} concentration was only marginally elevated by the toxin. The slight increase in cytosolic Ca^{2+} could be due to a low Ca^{2+} permeability of the channel, but it could also be due to release of Ca^{2+} from intracellular stores in response to the reduction in cytosolic pH caused by influx of H^+ through the toxin-induced channel (Sandvig and Olsnes, 1988).

The observed toxin-induced conductance increase seems to be receptor dependent and not due to non-specific interaction with the membrane, since the response was saturable and since rat pituitary cells (GH₄ cells), which lack diphtheria toxin receptors, were unaffected by the toxin. Similarly, mouse L cells, which do not have diphtheria toxin receptors, are not permeabilized to monovalent cations by the addition of diphtheria toxin at low pH, even when the toxin is accumulated at the surface membrane by fusion to an alternative ligand (Stenmark *et al.*, 1988). The observed dose-response relationship of the induced inward current in Vero cells is in good agreement with previous binding studies (Olsnes and Sandvig, 1986).

The increased membrane conductance observed by exposing Vero cells with surface-bound diphtheria toxin to low pH is most likely due to induction of new ion channels in the cell membrane. This is supported by our outside-out and cell-attached patch recordings, which in toxin-treated cells disclosed a channel type clearly different from the endogenous channels in untreated cells. The toxin-induced channel had properties compatible with the

toxin-induced conductance increase seen in the whole cell recordings. The single channel current was inward at negative membrane potentials, with reversal close to 0 mV. The single channel conductance was ~30 pS and the channel activity was voltage independent. In comparison, the channel formed in the endoplasmic reticulum and in the bacterial membrane upon release of nascent peptide chains with puromycin has a conductance of 220 pS (Simon and Blobel, 1991, 1992). This channel was permeable not only to cations, but also to anions such as glutamate and HEPES.

The reason for the ion selectivity and much lower conductance of the diphtheria toxin channel could be that it is specifically constructed to assist in the translocation of a single protein, the diphtheria toxin A-fragment. The toxin channel could therefore have a much smaller diameter than that of the endoplasmic reticulum channel, which must be able to accommodate different proteins and, during the initiation of translocation, even a polypeptide loop. Also, the requirements for translocation efficiency are likely to be much higher in the case of the endoplasmic reticulum channel than in that of the toxin and it could therefore need a wider channel. It should also be borne in mind that there is no direct evidence that the A-fragment is indeed translocated through the toxin channel, which could have another, so far undiscovered, function in the translocation mechanism.

Recently, Beise *et al.* (1994) reported that tetanus toxin forms channels of similar conductivity to the diphtheria toxin channel described here. In contrast to the diphtheria toxin channel, that of tetanus toxin was also found to be permeant for divalent cations and for chloride.

The receptor-independent channels induced by diphtheria toxin or its B-fragment in artificial lipid membranes are different from the channel observed in the present study. In artificial membranes the intact toxin forms a channel mainly selective for Cl^- at acidic pH and with a single channel conductance of ~6 pS (Donovan *et al.*, 1981), whereas the single channel conductance of channels formed by the B-fragment alone is ~10 pS at 100 mM KCl (Kagan *et al.*, 1981). The relatively high conductance of the toxin-induced channel in Vero cells may be due to a different mechanism of channel formation. In Vero cells toxin translocation and the associated ion fluxes are clearly receptor-mediated (Stenmark *et al.*, 1988). The interactions between the two toxin fragments and the receptor are rather complex. The B-fragment is responsible for both the specific receptor binding and for the increased transmembrane ion fluxes associated with translocation (see Sandvig and Olsnes, 1991), but the binding properties are also influenced by the A-fragment (Mekada and Uchida, 1985). It is reasonable to assume that these complex interactions, particularly between the receptor and the B-fragment, may affect the channel properties.

Materials and methods

Cell culture

The Vero cell line was chosen due to its high density of diphtheria toxin receptors (Middlebrook *et al.*, 1978). These cells are derived from the kidney of the African green monkey (*Ceropithecus aethiops*) and are frequently used in studies of diphtheria toxin effects. In control experiments we used the GH₄ rat anterior pituitary cell line. Rodents lack receptors for diphtheria toxin (Pappenheimer, 1977). The cells were

grown as monolayer cultures under standard tissue culture conditions at 37°C (Sandvig *et al.*, 1986). The cells used for the recordings were seeded 1–3 days before the experiments in 35 mm culture dishes. Vero cells have gap junctions and to facilitate recordings from single, independent cells, the seeding density did not exceed 10 000 cells per dish.

Electrophysiology

We used the patch clamp method in the cell-attached, whole cell and outside-out modes (Hamill *et al.*, 1981). Patch electrodes were coated with Sylgard and fire-polished and had a resistance of 6–15 MΩ when filled. The electrodes were connected to standard recording equipment. The recorded membrane potential was adjusted for the junction potential between the intracellular solution and the bath solution. The recordings were performed at room temperature on the stage of an inverted microscope. The cells were exposed to low pH by pressure ejection (~10 kPa) of pH 4.5 solution through a micropipette (inner tip diameter ~2 µm) positioned 15–20 µm from the cell.

Solutions

Prior to an experiment the culture medium was replaced with the following solution: 150 mM NaCl, 5 mM KCl, 2.4 mM CaCl_2 , 1.3 mM MgCl_2 , 10 mM glucose, 0.02% BSA, buffered by 10 mM HEPES–NaOH to pH 7.4. In the low pH solution for the ejection pipettes the HEPES–NaOH was replaced with 10 mM Na-gluconate and the pH was adjusted to 4.5 with HCl. This solution was also used as electrode solution for the cell-attached recordings and as one of the recording solutions in the initial experiments. In the whole cell and outside-out recordings the internal solution contained 140 mM K-gluconate, 5 mM KCl, 1 mM MgCl_2 , 2 mM Mg-ATP, buffered by 5 mM HEPES–NaOH to pH 7.2.

Microfluorometry of fura-2-loaded cells

The Vero cells were loaded with the fluorescent Ca^{2+} indicator fura-2 by exposure to 5 µM fura-2/AM in the normal recording solution for 30–60 min at 37°C. After washing and further incubation for 30 min, the cells were mounted on an Olympus inverted microscope, forming part of the Olympus OSP-3 system, for dual excitation microphotometry. The excitation light was switched at 200 Hz between 340 and 380 nm and the emitted fluorescence was measured at 500 nm by a photomultiplier. The measurements were restricted to single cells using a pinhole diaphragm and the absolute Ca^{2+} concentration was estimated from the ratio between the emissions at the excitation wavelengths of 340 and 380 nm (Grynkiewicz *et al.*, 1985). Calibration was obtained by recording the fluorescence ratio for droplets of 20 mM PIPES–NaOH buffer (pH 6.8) containing 10 µM fura-2 and different ratios of Ca-EGTA/EGTA, chosen to give concentrations of free Ca^{2+} covering the physiological range (Harafuji and Ogawa, 1980).

Acknowledgements

We are grateful to Dr M.Lanzrein for critical reading of the manuscript. This work was supported by The Norwegian Cancer Society, The Norwegian Research Council, The Jahre Foundation and Torsteds legat.

References

- Beise, J., Hahnen, J., Andersen-Beckh, B. and Dreyer, F. (1994) *Arch. Pharm.*, **349**, 66–73.
- Blaustein, R.O., Koehler, T.M., Collier, R.J. and Finkelstein, A. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 2209–2213.
- Boquet, P. and Duflot, E. (1982) *Proc. Natl Acad. Sci. USA*, **79**, 7614–7618.
- Borochov-Neori, H., Yavin, E. and Montal, M. (1984) *Biophys. J.*, **45**, 83–85.
- Collier, R.J. and Kandel, J. (1971) *J. Biol. Chem.*, **246**, 1496–1503.
- Donovan, J.J. and Middlebrook, J.L. (1986) *Biochemistry*, **25**, 2872–2876.
- Donovan, J.J., Simon, M.I., Draper, R.K., Montal, M. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 172–176.
- Draper, R.K. and Simon, M.I. (1980) *J. Cell Biol.*, **87**, 849–854.
- Glick, B.S., Beasley, E.M. and Schatz, G. (1992) *Trends Biochem. Sci.*, **17**, 453–459.
- Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) *J. Biol. Chem.*, **260**, 3440–3450.
- Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) *Pflügers Arch.*, **391**, 85–100.
- Harafuji, H. and Ogawa, Y. (1980) *J. Biochem. (Tokyo)*, **87**, 1305–1312.

- Kagan,B.L., Finkelstein,A. and Colombini,M. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 4950–4953.
- Mekada,E. and Uchida,T. (1985) *J. Biol. Chem.*, **260**, 12148–12153.
- Middlebrook,J.L., Dorland,R.B. and Leppla,S.H. (1978) *J. Biol. Chem.*, **253**, 325–7330.
- Milne,J.C. and Collier,R.J. (1993) *Mol. Microbiol.*, **10**, 647–653.
- Misler,S. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 4320–4324.
- Misler,S. (1984) *Biophys. J.*, **45**, 107–109.
- Morris,R.E., Gerstein,A.S., Bonventre,P.F. and Saelinger,C.B. (1985) *Infect. Immun.*, **50**, 721–727.
- Moskang,J.Ø., Sandvig,K. and Olsnes,S. (1988) *J. Biol. Chem.*, **263**, 2518–2525.
- Naglich,J.G., Metherall,J.E., Russell,D.W. and Eidels,L. (1992) *Cell*, **69**, 1051–1061.
- Olsnes,S. and Sandvig,K. (1986) *J. Biol. Chem.*, **261**, 1553–1561.
- Olsnes,S. and Sandvig,K. (1988) In Frankel,A.E. (ed.), *Immunotoxins*. Kluwer Academic Publishers, Boston, MA, pp. 39–73.
- Olsnes,S., Kozlov,J.V., van Deurs,B. and Sandvig,K. (1991) *Semin. Cell Biol.* **2**, 7–14.
- Papini,E., Sandomá,D., Rappuoli,R. and Montecucco,C. (1988) *EMBO J.*, **7**, 3353–3359.
- Pappenheimer,A.M., Jr (1977) *Annu. Rev. Biochem.*, **46**, 69–94.
- Rapoport,T.A. (1992) *Science*, **258**, 931–936.
- Sandvig,K. and Olsnes,S. (1980) *J. Cell Biol.*, **87**, 828–832.
- Sandvig,K. and Olsnes,S. (1988) *J. Biol. Chem.*, **263**, 12352–12359.
- Sandvig,K. and Olsnes,S. (1991) In Alouf,J.E. and Freer,J.H. (eds), *Sourcebook of Bacterial Protein Toxins*. Academic Press, New York, NY, pp. 57–73.
- Sandvig,K., Tønnessen,T.I., Sand,O. and Olsnes,S. (1986) *J. Biol. Chem.*, **261**, 11639–11644.
- Schiavo,G., Poulain,B., Rossetto,O., Benfenati,F., Tauc,L. and Montecucco,C. (1992) *EMBO J.*, **11**, 3577–3583.
- Shiver,J.W. and Donovan,J.J. (1987) *Biochim. Biophys. Acta*, **903**, 48–55.
- Simon,S.M. and Blobel,G. (1991) *Cell*, **65**, 371–380.
- Simon,S.M. and Blobel,G. (1992) *Cell*, **69**, 677–684.
- Stenmark,H., Olsnes,S. and Sandvig,K. (1988) *J. Biol. Chem.*, **263**, 13449–13455.
- Südhof,T., DeCamilli,P., Niemann,H. and Jahn,R. (1993) *Cell*, **75**, 1–4.
- Zalman,L.S. and Wisniewski,B.J. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 3341–3345.

Received on March 11, 1994; revised on July 21, 1994