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PVP-containing solutions for analysis of divalent cation-dependent NMDA responses in *Xenopus* oocytes

Martin Raditsch, Veit Witzemann*

Abteilung Zellphysiologie, Max-Planck-Institut für medizinische Forschung, Postfach 103820, D-69028 Heidelberg, Germany

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Abstract The electrophysiological analysis of Ca^{2+}-conducting ion channels in *Xenopus* oocytes is difficult due to secondary intracellular effects induced by Ca^{2+}. In the presence of polyvinylpyrrolidone (PVP) membrane currents can be recorded in nominally divalent cation-free solutions. The Ca^{2+}-permeable recombinant NMDA receptors of the NR1/NR2A subtype were used as assay system and the results show that PVP has no effect on NMDA receptor-induced currents. Ca^{2+} and Ba^{2+} depress NMDA-induced currents at submillimolar concentrations probably by interfering with the Na^+/K^+ flux. This block is fully reversible as also observed for Mg^{2+} but shows in contrast no pronounced voltage dependence. PVP-containing solutions may be useful for the analysis of divalent cation-dependent ion channels.

Key words: Xenopus oocyte; NMDA receptor; Divalent cation; Polyvinylpyrrolidone; Membrane current

1. Introduction

The expression of poly(A)$^+$RNA as well as of recombinant cRNA in *Xenopus* oocytes is widely used to analyse the pharmacological and physiological characteristics of neurotransmitter receptors since they exhibit many properties as observed in their native environment. Recently Leonard and Kelso (1990) [1] injected poly(A)$^+$ RNA from rat brain and demonstrated that any detailed analysis of NMDA-evoked currents is hampered by the interference with the currents mediated by the endogenous Ca^{2+}-activated Cl^--channels ($I_{Cl(Ca)}$). In special cases this problem can be circumvented by employing the Ca^{2+} chelator BAPTA [2] or Cl^- channel blockers in order to reduce Cl^- currents. Replacing Ca^{2+} by other divalent cations, e.g. Ba^{2+} [1], can be used to prevent modulatory divalent cation effects. In addition Ca^{2+} could affect desensitization or modulate ion conducting properties by binding to cytoplasmic or extracellular sites [3–7]. Therefore, it is important to study receptor function in divalent-free solutions. However, whole cell measurements on oocytes can not be reliably monitored under such conditions because of rapidly developing leakage currents.

In order to measure neurotransmitter-induced current flow in nominal divalent-cation-free solutions we tested high molecular weight compounds such as polymerized carbohydrates, dextran, or polyvinylpyrrolidone, PVP. These substances have been used as serum substitutes, as protective agents in cell culture, in the isolation of subcellular fractions, for tissue preservation for ultrastructural studies [8] or to experimentally alter osmotic pressure [9]. The essentially inert material is characterised by an excellent biological compatibility and low toxicity. It does not penetrate the cell membrane and by increasing the viscosity and osmotic pressure may protect the oocyte membrane from injury resulting from changing ionic environments and stress from bath application of buffers and ligands.

We used the Ca^{2+}-permeable NMDA receptor as assay system and injected recombinant cRNA of the NR1 and NR2A subunits [10] to define conditions which allow recordings with no added divalent cations. The effects of PVP on current leakage of oocytes in presence and absence of divalent cations were compared and the results show that PVP can be used to keep oocytes in a stable condition that allows electrophysiological measurements in the nominal absence of divalent cations. Surprisingly, we find that Ca^{2+} and Ba^{2+} have strong blocking effects on NMDA channels expressed in oocytes which is not the result of some artificial effect of PVP but due to the interference of divalent cations with the ion flow through the channel.

2. Materials and methods

Glutamate, glycine, flufenamic acid, and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) were purchased from Sigma. Polyvinyl pyrrolidone 25 (PVP) was obtained from Serva. Complementary DNAs encoding the NMDA receptor subunits, NR1 and NR2A [10] were subcloned into pSP64T [11] derived vectors [12]. The genuine 5′ and 3′ untranslated regions were mostly removed. Plasmids were linearized 3′ of the poly(A) stretch. NMDA receptor subunit-specific cRNAs were synthesized in vitro using SP6- polymerase [11]. *Xenopus laevis* oocytes were injected with 50 nl of cRNA (approx. 0.5 mg/ml) and incubated at 19°C in modified Barth's medium containing penicillin and streptomycin. Four hours after cRNA injection, oocytes were treated with collagenase type I (Sigma) (1 mg/ml) to remove the follicle cell layer. Agonist-activated currents were recorded 24 h–120 h after injection at room temperature using a two-microelectrode voltage clamp. Oocytes were perfused continuously with normal frog Ringer (NFR), containing 1.8 mM $CaCl_2$, 115 mM NaCl, 2.5 mM KCl and 10 mM HEPES (4-(2-hydroxyethyl)-1- piperazinethanesulfonic acid) adjusted to pH 7.2 (NaOH) or a nominally divalent cation-free PVP Ringer solution (PVP-R) containing 2 mM PVP instead of divalent cations. The flow rate was 6 ml/min. PVP was dissloved in H_2O (4 mM), adjusted to pH 7.2 and diluted 1:1 with two times concentrated NFR without $CaCl_2$, containing 230 mM NaCl, 5 mM KCl and 20 mM HEPES adjusted to pH 7.2. Flufenamic acid (0.8 mM) was added to block the Ca^{2+}-activated Cl^- channels.

Current and voltage electrodes were filled with 3 M KCl and had resistances between 0.5 and 3 MΩ. Current responses were recorded on a Graphtec chart-recorder. The NMDA receptor- mediated currents were measured by bath application of the various agonist containing solutions to activate the NMDA receptor channels. The agonists, glutamate (100 μM) and glycine (10 μM) were dissolved in NFR or PVP-R. To record current reduction NMDA receptors were activated by agonists and current block was induced by switching to solutions containing agonists and divalent cations at varying concentrations. At least five independent experiments were performed for each Ca^{2+} or Ba^{2+} concentration.

*Corresponding author. Fax: (49) (6221) 486 459.

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Viscosities of PVP-containing solutions were determined with an Ostwald viscosimeter. Relative viscosities were calculated from the experimentally measured ratio of flow times at 22°C and the ratio of densities of the solutions with and without PVP.

3. Results

3.1. NMDA receptor-mediated responses in PVP-R

Xenopus oocytes were injected with NR1- and NR2A-specific cRNAs. When the oocyte was bathed in normal frog Ringer (NFR) containing 2 mM Ca^{2+}, the inward current showed an early transient component which is believed to be mainly due to $I_{Cl(Ca)}$ [1] before reaching a steady-state current (Fig. 1). In contrast, in the PVP divalent-free Ringer's (PVP-R) the inward current showed no transient component and the steady-state current was greatly increased in amplitude in the presence of

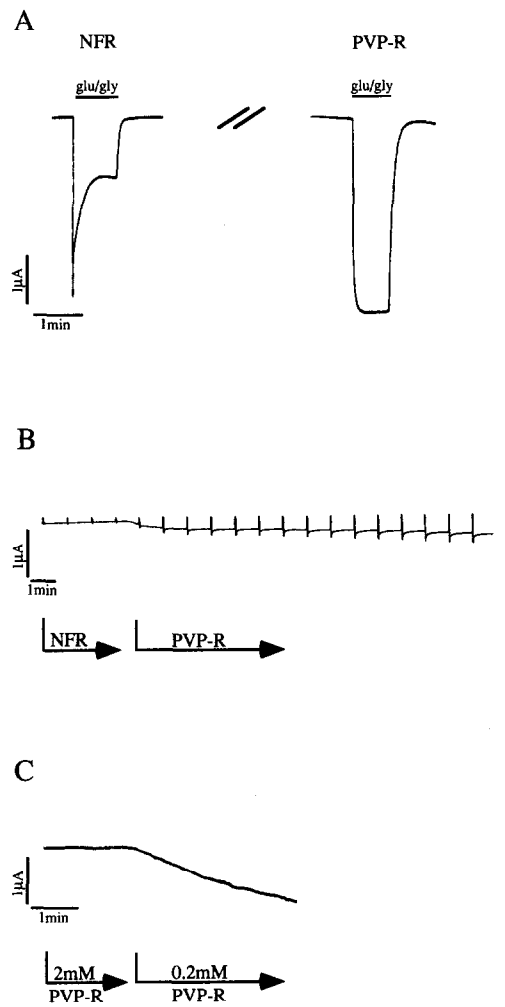

Fig. 1. NMDA receptor-mediated whole cell currents in NFR containing 2 mM Ca^{2+} or in nominally Ca^{2+}-free PVP-R. Oocytes were coinjected with 25 ng of NR1 and NR2A subtype cRNA and analysed within 1–5 days after injection. (A) Inward currents recorded at −70 mV upon application of 100 μM glutamate and 10 μM glycine in NFR containing 2 mM Ca^{2+} (left trace) or in nominally Ca^{2+}-free PVP-R medium (right trace). (B) Current trace to detect leakage currents in NFR or nominal Ca^{2+}-free PVP-R as indicated. Repetitive voltage steps from −70 mV to 0 mV were applied for 2 s every minute. (C) Leakage current of oocytes in PVP-R containing 2 mM and 0.2 mM PVP, respectively. Membrane voltage was clamped at −70 mV.

Ca^{2+} and remained stable throughout the application of agonist.

The stabilizing effect of PVP can be shown by comparing leakage currents in oocytes in NFR or PVP-R which were recorded over a period of 20 min with 2 s voltage steps (−70 mV to 0 mV) applied every minute. When switching from NFR to PVP-R with no Ca^{2+} added the leakage currents increased slightly but remained at a relatively low level (Fig. 1B). In Ca^{2+}-free NFR, however, leakage currents would increase dramatically within a few minutes precluding any reliable membrane current recording (not shown).

In *Xenopus* oocytes the time resolution of whole cell recordings in NFR is not sufficient to detect fast activation or inactivation changes that occur in the second range. In PVP-R the time for currents to reach steady state may be slower due to the higher relative viscosity, $\eta = 2.282$, for PVP-R containing 2 mM M_r 25,000 PVP. Higher concentrations, increasing the viscosity, resulted in significantly slower flow rates which were not suitable to record ligand-induced current traces. For this reason we did not test solutions containing mM concentrations of the larger M_r 40,000 PVP. To test whether the viscosity could be lowered to improve the current time course we measured the leakage current at reduced M_r 25,000 PVP concentrations. At 1 mM (relative viscosity $\eta = 1.527$) stable current measurements may be obtained (not shown) but at 0.5 mM (not shown) or at 0.2 mM leakage currents increased significantly (Fig. 1C). Viscositiy could be reduced also by replacing M_r 25,000 PVP by the smaller M_r 10,000 PVP. At 5 mM M_r 10,000 PVP ($\eta = 1.549$) viscosity was similar as in 1 mM M_r 25,000 PVP and both 'low viscous' solutions may be used even if the oocytes appeared to be less stable. At 2 mM M_r 10,000 PVP ($\eta = 1.169$), however, leakage currents were again increased precluding reliable current measurements. In all subsequent PVP experiments described here, M_r 25,000 PVP, at 2 mM concentration was used.

NMDA responses could be evoked repeatedly and in a reproducible fashion under PVP-R conditions (see Fig. 2). Some variation may be observed with respect to the current amplitudes which slowly decreased after repeated and prolonged activation of the NMDA channels. Such a current 'run down' may depend on receptor concentration, Ca^{2+} concentration, or simply on the condition of the isolated oocytes and is thus not attributable to the PVP buffer system. PVP, however, will not help to rescue already leaky oocytes or oocytes which have been kept in low Ca^{2+} conditions for extended time periods.

3.2. PVP does not change the NMDA receptor response

The increased currents seen in Fig. 1A could result either from the presence of PVP or, alternatively, currents observed in NFR could be reduced by Ca^{2+}. Fig. 2 A shows that the transient $I_{Cl(Ca)}$ were also detected in PVP-R containing 2 mM Ca^{2+} and the steady-state component showed no significant difference in comparison to the currents observed in NFR. However, in the absence of Ca^{2+}, using PVP-R, the current amplitude was increased without displaying an initial transient response. In the presence of the Cl^- channel blocker, flufenamic acid [1], the transient $I_{Cl(Ca)}$ was almost completely absent. In nominal Ca^{2+}-free PVP-R and in the presence of flufenamic acid, agonist-evoked current amplitudes were 3 to 4 times higher than in Ca^{2+}-containing buffer. These results suggest that Ca^{2+} present in NFR or PVP-R depressed the NMDA

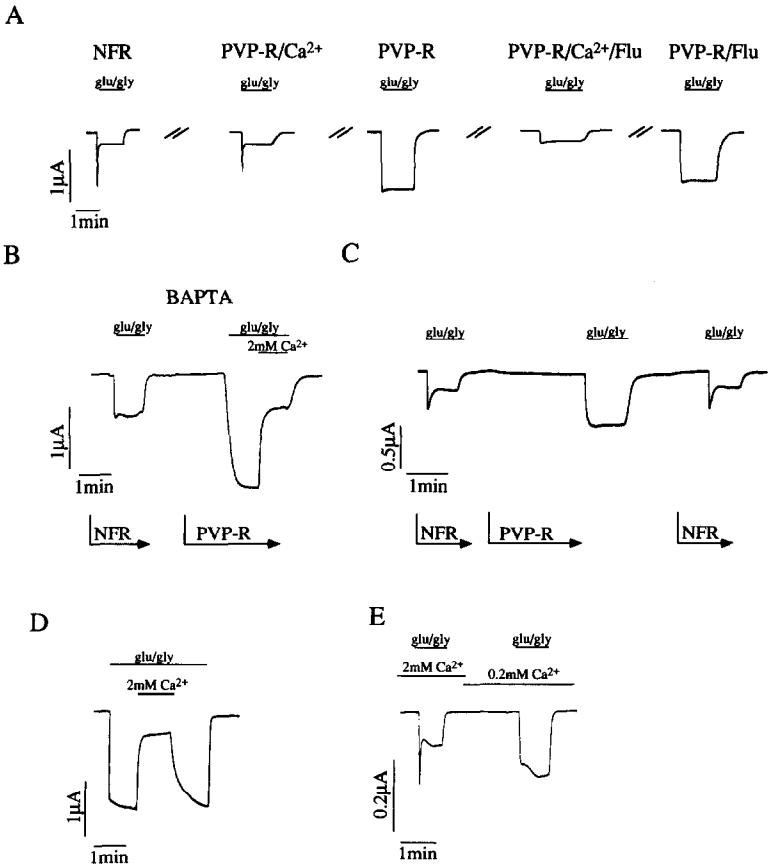

Fig. 2. Ca^{2+}-dependent currents mediated by NMDA receptors are not altered by PVP. Oocytes expressing NR1/NR2A receptors were analysed in NFR or PVP-R. (A) Glutamate/glycine responses were recorded in the absence or presence of the Cl^- current-blocking agent flufenamic acid (0.8 mM) and recordings were obtained from the same oocyte. Flufenamic acid (Flu) was used to block $I_{Cl(Ca)}$. (B) Oocytes were injected with BAPTA to prevent intracellular increases in Ca^{2+} and the activation of Cl^- currents [2]. BAPTA (50 nl, 25 mM) was injected as described [2] and prevented the intracellular increase in Ca^{2+}. In PVP-R in nominally Ca^{2+}-free conditions current amplitude was incrased as in (A). Application of 2 mM Ca^{2+} reduced currents by 80 (±6)% to the same level seen in NFR. (C) Stability and reproducibility of current responses in PVP-R. Continuous current trace from an oocyte expressing recombinant NR1/NR2A receptor subtypes which were activated by applying glutamate/glycine (100 μM/10 μM) in the Ca^{2+}-containing NFR. After wash out of the agonists, PVP-R was added. The small reversible shift in holding current may be due to differences in ion strength and osmotic pressure comparing NFR and PVP-R. (D) Ca^{2+}-dependent depression of NMDA response is reversible. Receptors were activated by agonists without Ca^{2+}. Upon addition of 2 mM Ca^{2+} current was reduced by about 80%. Initial current was recovered after removal of Ca^{2+}. (E) Amplitude of NMDA receptor-mediated currents in NFR depends on Ca^{2+} concentration. The continuous current trace was recorded as in (A). NMDA receptors were activated in the presence of 0.2 mM Ca^{2+} eliciting only small $I_{Cl(Ca)}$. Activation in NFR in the presence of 2 mM Ca^{2+} reduced the steady state current by about 50%. Switching back to low Ca^{2+} conditions demonstrated that block was reversible and depended on Ca^{2+} but not PVP.

response. To test whether this was caused by increasing intracellular Ca^{2+} concentrations, BAPTA, a Ca^{2+} chelator [1] was injected. As shown in Fig. 2B, BAPTA actually prevented $I_{Cl(Ca)}$ in NFR. It had, however, no effect on the increase of the current amplitude when NMDA responses were evoked by agonists in PVP-R with no Ca^{2+} added and could not prevent the current depression observed when switching to solutions containing in addition 2 mM Ca^{2+}. These results support the view that Ca^{2+} interacts either at sites accessible from the extracellular phase or directly interferes with the flow of ions through the channel. Furthermore, the Ca^{2+}-dependent effects were reversible when switching from Ca^{2+}-containing solutions to Ca^{2+}-free PVP-R and vice versa (Fig. 2C) or applying agonists with and without Ca^{2+} (Fig. 2D).

The dependence of the current amplitude of the NMDA responses on external Ca^{2+} also occurs in solution without PVP (Fig. 2E). Activation of the same oocyte in NFR containing 0.2 mM Ca^{2+} or 2 mM Ca^{2+}(amplitudes decrease 50%) revealed similar differences in steady state currents as observed in PVP-R and the Ca^{2+}-dependent depression was reversible. These results demonstrate that changes in ion conductance were not induced by PVP but rather due to the blocking action of Ca^{2+}. The Ca^{2+}-dependence of the NMDA response is not restricted to recombinant NR1/NR2A receptors but was also observed upon expression of rat brain poly(A)$^+$RNA (not shown).

3.3. *Ca^{2+}- and Ba^{2+}-dependent inhibition of whole cell currents*

By using PVP solutions containing flufenamic acid we were able to quantify the blocking actions of divalent cations in more detail. Fig. 3A shows that the blocking action of Ca^{2+} is weakly voltage-dependent. At −70 mV application of 2 mM Ca^{2+} decreased the current amplitude by 80% and at −30 mV by 70%. At positive membrane potentials there was also a reduction of the outward current amplitude by about 40%. Similar effects

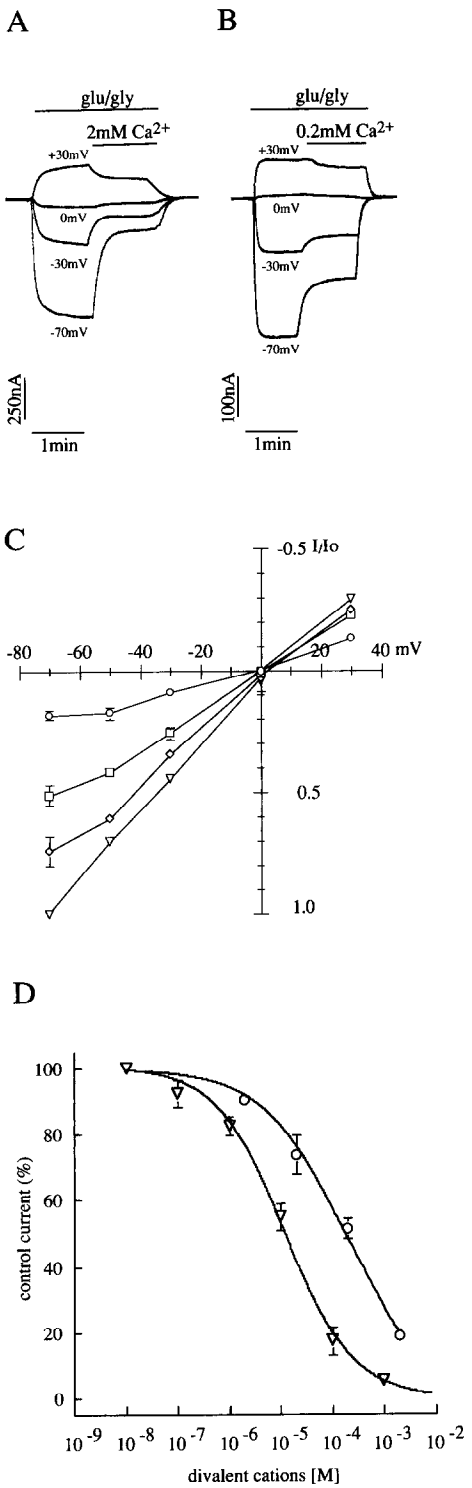

Fig. 3. Current–voltage dependence of Ca^{2+}-dependent block. NR1/NR2A receptors were activated by application of glutamate/glycine (100 μM/10 μM) in PVP-R containing flufenamic acid (1 mM) to block $I_{Cl(Ca)}$. (A) Currents recorded in presence of 2 mM Ca^{2+} at different membrane potentials. (B) Currents recorded in presence of 0.2 mM Ca^{2+} at different membrane potentials. (C) Current–voltage curves were obtained from individual recordings at the various membrane potentials and Ca^{2+} concentrations (▽ 0 μM; ◇ 20 μM; □ 200 μM; ○ 2 mM). The relative current is shown by the quotient I/I_0 according to the glu/gly evoked current at −70 mV (I_0). (D) Dose–response curve for Ca^{2+} and Mg^{2+} NR1/NR2A receptors were activated as in (A) in the presence of increasing Ca^{2+} (○) or Mg^{2+} (▽) concentrations at −70 mV. The apparent inhibition constants were 170 μM and 11 μM, respectively. Values represent means ± S.E.M. of 4 oocytes and data were fitted by the Cheng–Prusoff equation [18].

were observed at 0.2 mM Ca^{2+} concentrations (Fig. 3B). The *I–V* curves displayed only small shifts of the reversal potential around 0 mV (Fig. 3C) and only a weak voltage-dependence of the Ca^{2+} concentration-dependent depression.

Since NMDA receptors are permeable to Ba^{2+} [13] we measured the NMDA responses using PVP-R and flufenamic acid at different Ba^{2+} concentrations and membrane potentials. As observed for Ca^{2+}, Ba^{2+} reduced currents in a concentration-dependent manner displaying again no pronounced voltage dependence (Fig. 4). This is in contrast to Mg^{2+} which blocks the NMDA response in NFR or PVP-R in a strongly voltage-dependent manner (not shown) and at significantly lower concentrations as illustrated by the dose–response curves in Fig. 3C.

The finding that Ba^{2+} can replace Ca^{2+} favours the view that the depression of the NMDA response is mainly due to an interference with other ions flowing through the channel. Alternatively, Ca^{2+} could affect agonist binding and agonist-dependent desensitization by interacting with extracellularly exposed sites. In order to test whether Ca^{2+} could modulate the agonist potencies at steady state conditions, dose–response curves for glutamate and glycine were measured in the presence (0.2 mM) or nominal absence of Ca^{2+}. In PVP-R containing flufenamic acid, Ca^{2+} had no apparent effect on the binding properties of the two ligands and the apparent EC_{50} for glutamate was 2 μM with no Ca^{2+} added or 3 μM at 0.2 mM Ca^{2+}. For glycine the EC_{50} values were 2.5 μM at both conditions (not shown).

4. Discussion

A serious limitation of the *Xenopus* oocyte expression resides in the apparent requirement for Ca^{2+} or partly substituting divalent cations such as Ba^{2+} to prevent the development of leakage current. Additional difficulties are caused by Ca^{2+}-meditated changes in receptor function and by secondary effects such as Ca^{2+}-activated endogenous Cl^- channels [1]. Therefore it is not only difficult to analyse the agonist-induced current from the Ca^{2+}-triggered responses but it is not possible to discriminate in whole cell experiments between different actions of divalent ions on the conductance of Ca^{2+}-permeable ion channels.

In search of conditions which allow electrophysiological measurements on oocytes in nominally Ca^{2+}-free solutions we tested high molecular weight compounds which could stabilize membrane current recordings without interfering with the physiological response of ion channels. Adding the M_r 25,000 PVP at a concentration of 5% (2 mM) to the bathing medium effectively stabilized oocyte recordings in divalent cation-free media. These solutions have a relative viscosity of $\eta = 2.282$ compared to NFR and may represent the best compromise choosing between low leakage currents and practicable flow rates for bath application of ligands. In order to increase flow

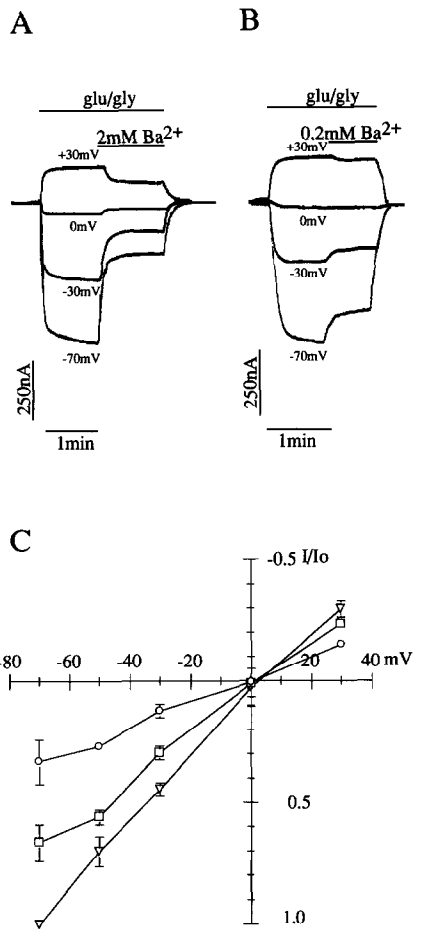

Fig. 4. Current–voltage dependence of Ba^{2+}-dependent block NR1/NR2A receptors were activated by application of glutamate/glycine (100 μM/10 μM) in PVP-R containing flufenamic acid (1 mM). (A) Currents recorded in presence of 2 mM Ba^{2+} at different membrane potentials. (B) Currents recorded in presence of 0.2 mM Ba^{2+} at different membrane potentials. (C) Current–voltage curves were obtained from individual recordings at the various membrane potentials as shown in (A) and (B). The Ba^{2+}-dependent inhibition of the agonist response displayed no pronounced voltage-dependence (▽ 0 μM; □ 200 μM; ○ 2 mM).

rates M_r 25,000 PVP at 1 mM concentrations or M_r 10,000 PVP at 5 mM concentrations may be used although at the expense of oocyte stability. Dextran, a neutral biopolysaccharide of M_r 40,000–60,000 (at 5%), with similar properties as PVP, had no obvious protective effect (not shown) and the efficiency of higher dextran concentrations was not tested.

Oocytes in the PVP-R remained in a stable condition and electrophysiological measurements could be performed without the development of significant leakage currents over a period of 30 min to 1 h. The quality of the recordings depends of course on the condition of the oocytes, i.e., the resting potential (ca. −30 mV) and mechanical stability of the oocyte membranes varies comparing different oocyte batches and often decreases with longer incubation times. Thus oocytes 1 to 2 days after injection are usually most suitable for analysis. The beneficial effect of PVP-containing media may be due to the increased osmotic pressure and viscosity which apparently stabilizes the oocyte membrane without interfering with the bath application of ligands and ion translocation. PVP, however, is not meant to replace NFR but offers a possibility to record current traces in divalent-free solutions. PVP will not improve recordings from poor quality oocytes or from oocytes which have already developed leaks due to extended incubation at low divalent cation concentration.

PVP when added to NFR had no effect on agonist-evoked currents and the Ca^{2+}-induced Cl^- currents could be blocked by flufenamic acid. However, when currents were induced in nominally Ca^{2+}-free PVP-R the amplitudes were increased 3–4-fold which was not due to PVP but depended on the Ca^{2+} concentration. Thus PVP-R provides a basis to analyse separately the interaction between Ca^{2+} or other divalent cations and NMDA receptors. The apparent affinities of glutamate or glycine at steady state conditions were not changed at 0.2 mM Ca^{2+} concentrations indicating that neither PVP nor Ca^{2+} alter the apparent potencies of the agonists. These results may also indicate that neither PVP nor the increased viscosity affect the behaviour of the channel, a view, which is supported by single channel recordings of Ca^{2+}-activated K^+channels where the kinetics of channel gating were not changed when increasing the viscosity of the external medium 2.6-fold [14].

The depression of the NMDA response in oocytes observed in PVP-assay conditions supports the view that Ca^{2+} reduces ion flow through the channel: with Ca^{2+} concentrations increasing from 0 to 2 mM Ca^{2+} the inward current decreased at −70 mV by about 80% and the outward current at +30 mV by about 40%. The observation that Ca^{2+} reduced outward currents could be explained by the fact that at +20 to +30 mV there is still a significant Ca^{2+} influx [15] which is sufficient to interfere with the outward current. The Ca^{2+}-dependent changes showed neither a pronounced voltage dependence nor a dramatic shift in reversal potential which is in contrast to the strongly voltage-dependent channel block by Mg^{2+} ions [16]. The complete reversibility of the Ca^{2+}-dependent changes and the fact that a similar depression of the steady state current occured in the presence Ba^{2+} suggests that these divalent cations interfere with the conductance of other ions as suggested by [13,15,16]. Additional, rapidly occurring desensitizing effects of extracellular Ca^{2+} [6,17] could not be resolved in our whole cell measurements but may be responsible for the stronger Ca^{2+} effect (ca. 50% current block at 0.2 mM Ca^{2+}) observed here as compared to the reduction of the single channel conductance (ca. 30% at 0.2 mM Ca^{2+}; [15]). An internal action which has been observed in nerve cells [3–5,7] is unlikely since the Ca^{2+}-dependent depression was prevented by injection of BAPTA.

Using the NMDA response we show that membrane currents can be reliably recorded, with no divalent cations added, in oocytes stabilized by PVP-containing solutions . It is possible to measure dose response curves for individual divalent cations. These assay conditions will therefore be useful to further analyse the putative multiple actions of divalent cations and their competition with other permeating ions or channel blockers on the NMDA receptors. Similarly, PVP-R may be useful for the analysis of other Ca^{2+}-permeable ion channels such as the neuronal acetylcholine receptors or Ca^{2+} channels.

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