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Journal of Neuroscience Methods 153 (2006) 62–70

JOURNAL OF NEUROSCIENCE METHODS

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Fluctuations in *Xenopus* oocytes protein phosphorylation levels during two-electrode voltage clamp measurements

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Received 7 May 2005; received in revised form 7 October 2005; accepted 9 October 2005

Abstract

The biophysical and pharmacological properties of ion channels and transporters are often studied in exogenous expression systems using either the two-electrode voltage clamp (TEVC) in *Xenopus* oocytes or the patch clamp techniques. Cells machinery is trusted to produce active proteins that are correctly phosphorylated and glycosylated. However, native physiological cellular processes that might be altered during the course of the experiment are often ignored. Here, we detected and quantified the effects of various electrophysiological recording conditions on the phosphorylation levels of *Xenopus* oocytes proteins, including membrane proteins, as phosphorylation/dephosphorylation events modulate ion channels gating and cell surface expression.

Two strategies were chosen to determine relative protein phosphorylation levels: a direct detection with a phospho-Ser/Thr PKA substrate antibody, and a functional method employing two different leak potassium channels as indicators, chosen based on their opposite responses to protein kinase phosphorylation. We report that holding potential, and bath solution properties such as pH, osmolarity, temperature and ion composition, dramatically affect protein phosphorylation levels in *Xenopus* oocytes. Our results might explain some of the fluctuations in the biophysical properties of expressed channels, often observed during electrophysiological measurements.

Minimizing possible misinterpretations could be achieved using either mutated, kinase insensitive, channels or kinases/phosphatases modulators.
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Keywords: TEVC; Current fluctuations; Ion channels; Phosphorylation; Protein kinases; Protein phosphatases; PKA

1. Introduction

The two-electrode voltage clamp electrophysiological recording technique is often applied for the study of ion channels and transporters expressed in *Xenopus laevis* oocytes (Stuhmer and Parekh, 1995). Based on the pioneering work of Gurdon et al. (1971), who discovered that *Xenopus* oocytes can translate injected foreign RNA into proteins, Gundersen et al. (1983a,b) and Miledi et al. (1983) reported the successful expression of functional receptors and channels in *Xenopus* oocytes. Combined with our ability to routinely clone and mutate a variety of ion transporting proteins, this method has evolved into a powerful tool in the study of ion channels.

Xenopus oocytes were chosen as a preferred model system as cells are easily obtained, large and hardy, can tolerate repeated impaling by microelectrodes, have only few endogenous channels, and readily express foreign mRNA (Stuhmer and Parekh, 1995). Indeed, among the many different electrophysiological techniques that have been described, TEVC recording from *Xenopus* oocytes seems particularly suitable for the implementation of automated measurement systems (Pehl et al., 2004).

After foreign mRNA injection, to explore channel characteristics, oocytes are held at various membrane potentials and exposed to a variety of external conditions. Routinely, oocytes are measured under "physiological" conditions: experiments are done at room temperature (20–25 °C) at a holding potential of −80 mV and the external bath solution contains (in mM) 4 KCl, 96 NaCl, 1 $MgCl_2$, 0.3 $CaCl_2$ at pH 7.5. Nevertheless, each of the external conditions is frequently changed according to the experiment type. Expressed channels are frequently tested for pH, temperature or osmolarity sensitivity while shifting the appropriate variables. Furthermore, oocyte holding potential is frequently changed to match the expressed channel Nernst potential; external potassium concentration is often increased to allow measurement of inward currents; bath solution cation composition is varied to address selectivity issues.

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0165-0270/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2005.10.005

Interestingly, the influence of these variable recording conditions on oocytes themselves, and hence, their indirect effects on the examined channel, is often neglected. Possible effects might include a shift in activity levels of protein kinases and phosphatases, which can result in a change in the phosphorylation status of membranal proteins, including that of the channel of interest.

In an increasing number of reports, the addition of a negative charge on serine, threonine or tyrosine residues, achieved by protein phosphorylation, has been shown to dramatically affect ion channel properties (Catterall, 2000; Levitan, 1994; Soderling, 1995). Following are only a few of the changes in channel properties that have been found to be due to protein phosphorylation: The activity of the L-type (dihydropyridine-sensitive) voltage-dependent Ca^{2+} channels in the heart and skeletal muscle is strongly enhanced by epinephrine and norepinephrine, mainly via the cAMP protein kinase A (PKA) pathway (Hartzell et al., 1991; Trautwein and Hescheler, 1990; Reuter, 1983; Perets et al., 1996). Phosphorylation of the Kv1.1/Kvβ1.1 (αβ) K1 α subunit at Ser-446 increases A-type activity by enhancing both channel inactivation and current amplitude (Levin et al., 1996). Bidirectional synaptic plasticity events are known to be mediated by phosphorylation of the AMPA receptor subunit, GluR1, at two sites on its intracellular carboxy-terminal domain (Lee et al., 2000). Phosphorylation of the two different sites allows control over the open probability of the channel as well as the apparent single-channel conductance of the receptor (Banke et al., 2000; Derkach et al., 1999). Calcium–calmodulin-dependent kinase II (CAMKII) modulates Kv4.2 channel expression and upregulates neuronal A-type potassium currents (Varga et al., 2004). Treatment of cultured embryonic rat brain neurons with protein kinase C (PKC) activators slows the time course of inactivation of macroscopic sodium current (Numann et al., 1991). Phosphorylation influences neurosteroid modulation of synaptic $GABA_A$ receptors in rat CA1 and dentate gyrus neurons (Harney et al., 2003). The different subunits of the NMDA receptor are under tight regulation by various kinases that modulate the receptor sensitivity to ethanol (Ron, 2004). Protein phosphorylation regulates BK channel activity in rat cerebellar Purkinje neurons (Widmer et al., 2003). Anchoring proteins function is also regulated by phosphorylation. The targeting domain of the A-kinase anchoring protein, AKAP79, contains putative phosphorylation sites for PKA and PKC, which regulates its binding to membrane vesicles (Dell'Acqua et al., 1998).

Fluctuations in phosphorylation levels of expressed membrane proteins might explain variations in the biophysical properties of expressed channels, often seen during electrophysiological measurements. We encountered this current instability in some of our previous work with members of the potassium leak channels. The activity of those channels is known to be highly regulated by various pathways including protein phosphorylation (Goldstein et al., 2001; Patel and Honore, 2001). Therefore, we examined whether fluctuations in protein phosphorylation levels could account for the observed phenomenon in *Xenopus* oocytes.

Here, we studied the effects of commonly used experimental conditions, such as holding potential, bath solution, ion composition, temperature, pH and osmolarity, on phosphorylation levels of *Xenopus* oocytes proteins in general and expressed membranal proteins in particular. Two strategies were chosen to determine relative phosphorylation levels: the first employs a phospho-Ser/Thr PKA substrate antibody for a direct detection, while the second employs two different potassium leak channels as indicators, chosen based on their opposite responses to protein kinase phosphorylation. We report that common conditions, frequently used in ion channel electrophysiological recording procedures, dramatically affect phosphorylation levels in *Xenopus* oocytes. Possible misinterpretations of channels functional data could be minimized by using either mutated, kinase insensitive channels or kinase/phosphatase modulators.

2. Materials and methods

2.1. Molecular biology

The wt-KCNK0 *Drosophila* channel clone and its deletion mutant, KCNK0Δ299-1001, were cloned downstream to a T7 RNA polymerase promoter as described previously (Goldstein et al., 1996; Goldstein et al., 1999; Zilberberg et al., 2000). Wt-KCNK2 as well as its variants, KCNK2-S348A and KCNK2-S348D were cloned into pRAT plasmid as previously described (Bockenhauer et al., 2001). cRNA was transcribed using T7 polymerase and the mMESSAGE mMACHINE^{TM} kit (Ambion, Austin, TX).

2.2. Immunoblot analysis

Proteins were extracted from un-injected oocytes 1–3 days after isolation. Oocytes were homogenized in groups of five by vortexing in 150 μl homogenization buffer (0.1 M NaCl, 1% Triton X-100, 1 mM PMSF, 10 mM L-methionine, 20 mM Tris–HCl, pH 7.6, 1 mM sodium pyrophosphate, 50 mM β-glycerophosphate, 6 mM 4-nitrophenyl phosphate, 1 mM sodium vanadate, supplemented with 1:1000 protease inhibitor cocktail for general use). In the holding potential experiments, each oocyte was held at either −80 or −20 mV for 5 min, placed in a tube containing 30 μl homogenization buffer and immediately frozen by liquid nitrogen to maintain the holding potential effect. Oocytes were homogenized while defrosting.

Protein concentrations were determined with the Bradford method (Bradford, 1976): 12 μg of total protein extract was separated on 11% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, Maidstone, England). Protein quantities were slightly readjusted to give a uniform staining with Ponceau Red (not shown). Membranes were blocked with 5% non-fat dry milk and 0.1% Tween-20 in TBS (Tris-buffered saline) for 1 h at room temperature and washed quickly three times with 0.1% Tween-20 in TBS. Membranes were then incubated with phospho-(Ser/Thr) PKA substrate antibody (Cell Signaling, Beverly, MA), diluted 1:1000 in TBS with 0.1% Tween-20 and 5% BSA for 16 h at 4 °C. Three 5 min washes were performed prior to a 1 h incubation with goat anti-rabbit IgG HRP-conjugated antibodies (ZyMax^{TM}, San-Francisco, CA) diluted 1:10,000 in 0.1% Tween-20 in TBS containing 5% non-fat dry

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