Modulation of Kv3 potassium channels expressed in CHO cells by a nitric oxide-activated phosphatase

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1. Voltage-gated K^+ channels containing Kv3 subunits play specific roles in the repolarization of action potentials. Kv3 channels are expressed in selective populations of CNS neurons and are thought to be important in facilitating sustained and/or repetitive high frequency firing. Regulation of the activity of Kv3 channels by neurotransmitters could have profound effects on the repetitive firing characteristics of those neurons.

2. Kv3 channels are found in several neuronal populations in the CNS that express nitric oxide synthases (NOSs). We therefore investigated whether Kv3 channels are modulated by the signalling gas nitric oxide (NO).

3. We found that Kv3.1 and Kv3.2 currents are potentially suppressed by D-NONOate and other NO donors. The effects of NO on these currents are mediated by the activation of guanylyl cyclase (GC), since they are prevented by Methylene Blue, an inhibitor of GC, and by ODQ, a specific inhibitor of the soluble form of GC. Moreover, application of 8-Br-cGMP, a permeant analogue of cGMP, also blocked Kv3.1 and Kv3.2 currents.

4. KT5283, a cGMP-dependent protein kinase (PKG) blocker, prevented the inhibition of Kv3.1 and Kv3.2 currents by D-NONOate and 8-Br-cGMP. This indicates that activation of PKG as a result of the increase in intracellular cGMP levels produced by D-NONOate or 8-Br-cGMP is necessary for channel block.

5. Although the effects of NO on Kv3.1 and Kv3.2 channels require PKG activity, two observations suggest that they are not mediated by phosphorylation of channel proteins: (a) the reagents affect both Kv3.2 and Kv3.1 channels, although only Kv3.2 proteins have a putative PKA–PKG phosphorylation site, and (b) mutation of the PKA–PKG phosphorylation site in Kv3.2 does not interfere with the effects of NO or cGMP.

6. The inhibitory effects of NO and cGMP on Kv3.1 and Kv3.2 currents appear to be mediated by the activation of serine–threonine phosphatase, since they are blocked by low doses of okadaic acid. Furthermore, direct intracellular application of the catalytic subunit of protein phosphatase 2A inhibited Kv3.2 currents, indicating that activity of PKG-induced phosphatase is necessary and sufficient to inhibit these channels.

7. The results suggest that basal phosphorylation of Kv3 channel proteins is required for proper channel function. Activation of phosphatases via NO or other signals that increase cGMP might be a potent mechanism to regulate Kv3 channel activity in neurons.

Potassium channels constitute a large and diverse group of ion channels which play important roles in the generation and transmission of electrical signals (Llinas, 1988; Rudy, 1988; Hille, 1992; Coetzee *et al.* 1999). A distinctive combination of K^+ channels endows neurons with a broad repertoire of excitable properties and allows each neuron to respond in a specific manner to a given input at a given time (Llinas, 1988; Hille, 1992). In addition, the properties of many K^+ channels can be modulated by second-messenger pathways activated by neurotransmitters and other stimuli. In fact K^+ channels are among the most frequent targets of the actions of several signalling systems (Levitan, 1988; Hille, 1992; Reinhart & Levitan, 1995). The resulting dynamic, electrophysiological identity of

neurons contributes to the complexity of electrical signalling in the nervous system.

A large number of K^+ channel proteins were recently discovered using molecular analysis (Coetzee *et al.* 1999). Some of these proteins are pore-forming subunits of K^+ channels previously known from electrophysiological studies. However, the native counterparts and physiological role of many of the cloned subunits are unknown and remain to be established. Among the cloned subunits, the members of the Kv3 subfamily (which include the products of four genes known as *Kv3.1–Kv3.4*) have generated special interest. *Kv3* genes are members of one of the subfamilies of the Kv family of K^+ channel genes, which encode pore-forming subunits of voltage-gated K^+ channels. Kv3 sububnits express channels which have unusual electrophysiological properties including a very positive activation voltage and very fast deactivation rates (Rudy *et al.* 1999). In addition, they have intriguing and specific patterns of expression in the brain (Weiser *et al.* 1995; Rudy *et al.* 1999). Given their unique electrophysiological properties and their patterns of expression in the CNS, it is believed that these channels play special roles in neuronal excitability. One of these roles appears to be fast spike repolarization and facilitation of sustained high frequency firing in fast spiking cortical interneurons and central auditory processing neurons (Wang *et al.* 1998; Erisir *et al.* 1999; Lau *et al.* 2000). However, other roles are possible since Kv3 channels are also expressed in neurons that do not fire at high frequencies.

Kv3 channels are usually expressed in neurons that also contain other voltage-activated K^+ channels (Coetzee *et al.* 1999; Rudy *et al.* 1999). When different types of K^+ channels are present in a single cell, the selective modulation of a given channel type by second messenger systems changes the channel properties and/or its relative contribution to the total K^+ current. This differential modulation could bestow the cell with the ability to modify different aspects of its electrical behaviour. Therefore, in order to understand the specific roles of Kv3 channels in neuronal excitability, it is important to investigate their modulation by neurotransmitters and other neuromodulators.

Among the various neuromodulators that, given the distribution of Kv3 gene products in the brain, could regulate Kv3 channel function in neurons, we decided to investigate nitric oxide (NO). NO belongs to a novel class of messenger molecules, membrane permeable gases which act as diffusible signals for intra- and intercellular communication and are thought to be important modulators of neuronal function (Verma *et al.* 1993; Bredt & Snyder, 1994; Ko & Kelly, 1999). NO has been linked to a number of important functions in the CNS, including neuroprotection, synaptic transmission-related processes such as long-term potentiation (LTP) and long-term depression (LTD), synchronization of synaptic connections within locally defined areas of neuronal circuits, development, sleep regulation and arousal (Pape & Mager, 1992; Bredt & Snyder, 1994; Meffert *et al.* 1994). The enzymes that produce NO, the nitric oxide synthases (NOSs), are expressed in a broadly distributed but very small (2%) subset of neurons, indicating specific roles in a variety of brain functions (Vincent & Kimura, 1992). NOSs have been identified in neuronal cell bodies, as well as in nerve terminals, where NO is thought to influence neurotransmitter release (Bredt & Snyder 1994; Li *et al.* 1998). It is now clear that NO can exert its biological effects through the activation of guanylyl cyclases and the production of cGMP (Tsou *et al.* 1993; Pineda *et al.* 1996) or through cGMP-independent mechanisms known as 'redox signalling' (Stamler, 1994). However, relatively few targets of the action of NO in neurons that could explain its role in CNS functions have been identified.

Kv3 proteins are also expressed in the soma, axons and presynaptic terminals of selective neuronal populations in the CNS (Weiser *et al.* 1995; Moreno *et al.* 1995). Interestingly, these include some of the populations that express NOS most prominently. For example, both Kv3.1 and NOS proteins are abundant in cerebellar granule cells (Weiser *et al.* 1995) and in the mesopontine cholinergic system (Tyler *et al.* 1999). In addition, these cholinergic neurons project heavily to the thalamus, and it is in the thalamocortical neurons throughout the dorsal thalamus that Kv3.2 subunits are most prominantly expressed. Kv3.2 channels are also expressed in the terminals of NOS-containing cerebellar basket cells on Purkinje cells (Bobik *et al.* 1998). Other neuronal populations that express the channel proteins and either express NOS or receive inputs from NOS-containing cells include the retinoceptive layers of the superior colliculus, globus pallidus, basal forebrain and cortical interneurons (Weiser *et al.* 1995; Moreno *et al.* 1995). These findings suggest that Kv3 channels could constitute a target for the modulatory action of NO.

Given the interest in identifying targets of NO in CNS neurons, the apparent co-localization of NOS and Kv3 channels in several neuronal populations, and the presence of these channels in neurons that either receive input from or are under the potential influence of NO-producing cells, we asked whether Kv3 channels are possible targets of the NO signalling system. We investigated whether NO has the ability to modulate K^+ currents generated by Kv3.1 and Kv3.2 subunits in heterologous expression systems. The experiments have revealed that NO modulates the activity of these neuronal voltage-gated K^+ channels through a novel cGMP/PKG-mediated signalling cascade. Modulation of Kv3 channels by this cascade may be associated with their specific roles in the mammalian CNS.

METHODS

Preparation of CHO cells expressing Kv3.2 and Kv3.1 channels

For transient expression of Kv3.1 channels, cDNA encoding the Kv3.1b channel subunit (the major product of the *Kv3.1* gene; see Rudy *et al.* 1999) was subcloned in the expression vector pcDNA3.1+ (InVitrogen, CA, USA). CHO (Chinese hamster ovary) K1 cells (American Type Culture Collection, Bethesda, MD, USA) were plated at 60% confluence and cotransfected with green fluorescent protein (GFP; InVitrogen) and the channel cDNA, utilizing the lipofection method (DOTAP, Boehringer Mannheim) as described by the manufacturer. Fluorescent cells were recorded 48–90 h after transfection using an excitation wavelength of 488 nm and emission at 515 nm. The preparation of stably transfected CHO cells expressing th Kv3.2a product of the *Kv3.2* gene was as previously described (Moreno *et al.* 1995). Microinjection of mutant Kv3.2a (SS[563–564]AA) cRNA into CHO cells was performed as in Moreno *et al.* (1995). Cells were cultured as previously described (Moreno *et al.* 1995).

Electrophysiological analysis

Whole-cell currents were obtained at room temperature with the whole-cell configuration of the patch clamp technique (Hamill *et al.* 1981) in tissue culture dishes on the stage of an inverted microscope, using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). Unless otherwise indicated, patch pipettes were filled with a solution containing (mM): 130 KCl, 10 EGTA, 1 $MgCl_2$, 10 Hepes, 5 MgATP (pH adjusted to 7.4 with KOH) (normal solution) and had resistances of 2–4 MΩ. In some experiments MgATP was removed or the EGTA replaced by 15 mM EDTA in 0 $MgCl_2$. The extracellular solution contained (mM): 135 NaCl, 3.5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 5 glucose and 10 Hepes (pH adjusted to 7.4 with NaOH). Seal resistance was typically 10 GΩ. Recordings were made with partial series resistance compensation (70–80%) and most of the cell capacitance subtracted. In the experiments where PP1, PP2A or buffer was included in the intracellular solution of 'low resistance patch pipettes', the pipettes had resistances between 0.7 and 1.0 MΩ. 'High resistance pipettes' filled with intracellular solution containing PP2A or PP1 had resistances of 2–3 MΩ. Currents were low-pass filtered at 2 kHz using an eight-pole Bessel filter (Frequency Devices) and digitized at 2.5 kHz. Substraction of leak and remaining capacitance was obtained using a *P*/4 protocol. To generate voltage clamp protocols, and for data acquisition and analysis we used the pCLAMP 6 software (Axon Instruments).

Experiments with 8-Bromo-cGMP (8-Br-cGMP), 8-(4-chlorophenyl-thio)-cAMP (CPT-cAMP), and the different NO donors were performed only when recordings were stable. The compounds in extracellular solution were applied locally to the cell being recorded through a blunt pipette by gravity flow.

Single channel recordings utilized the inside-out configuration of the patch clamp technique (Hamill *et al.* 1981) with the same amplifier. CHO cells were superfused with (mM): 90 KMeSO4, 20 KCl, 5 $MgCl_2$, 10 Na-Hepes, 0.1 $CaCl_2$ and 15 glucose (pH 7.3). All patches were excised into this solution. In the experiments in which ATP was included in the solution, in order to maintain free $MgCl_2$ at the same concentration as in the control solution, the ATP-Mg, together with additional $MgCl_2$ was added based on calculated values obtained using the equation of Fabiato & Fabiato (1979). The pipette solution had the same composition as the extracellular solution used for the whole-cell experiments. The currents were low-pass filtered at 5 kHz. Leak subtraction was digitally performed using a template generated by averaging sweeps with no openings and this average subtracted from sweeps containing openings. Data were analysed utilizing pCLAMP software.

Preparation and use of specialized reagents

8-Br-cGMP, sodium orthovanadate, haemoglobin (Hb; bovine), okadaic acid, okadaic acid methyl ester, hydrogen peroxide and Methylene Blue, were purchased from Sigma or RBI. FK506 was obtained from Fujigawa (Japan); the catalytic subunit of cGMP-dependent protein kinase (PKG), CPT-cAMP and catalase were purchased from Boehringer Mannheim. 3-Morpholinosydnominine (SIN1) microcystin LR and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were purchased from RBI (Natick, MA, USA). 1,1-Diethyl-2-hydroxy-2-nitrosohydrazine (D-NONOate) was from Cayman Chemical (Ann, Arbor, MI, USA) and KT5823 and 1H[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) from Calbiochem–Navabiochem Corp. The catalytic subunits of PP2A (PP-2Ac) and PP1 (PP-1c) were a kind gift of Angus C. Nairn, (Rockefeller University). The enzymes were diluted 1:10 in intracellular pipette solution (0.05 M Tris-HCl, pH 7.0, 0.1 mM EGTA, 0.1% 2-mercaptoethanol and 60% v/v glycerol) from a stock of 0.9 and 4.2 u ml^{-1}, respectively, and stored at −20°C. All other reagents were from Sigma.

All the reagents to stimulate or inhibit the signalling enzymes were prepared fresh on the day of the experiment. 3-Isobutyl-1-methylxanthine (IBMX) was dissolved in water and then diluted to the desired concentration in extracellular solution. CPT-cAMP and 8-Br-cGMP were dissolved directly in extracellular solution. SIN-1 was dissolved in water at 10 mg ml^{-1} and then diluted in extracellular solution. The catalytic subunit of PKG was dissolved in the buffer provided by the supplier and then diluted to the desired concentration in the solution used for the intracellular side of inside-out patches (see below). SNAP, ODQ and KT5823 were first dissolved in dimethyl sulphoxide (DMSO) and then diluted in extracellular solution to the desired concentration with a final DMSO concentration of $< 0.1\%$. D-NONOate was dissolved in diluted NaOH (10 mM, pH ~10) to prevent the spontaneous release of NO, and then diluted with extracellular solution and pH adjusted to the desired value with diluted HCl for immediate use (pH 7.3, unless otherwise indicated). Okadaic acid, its methyl ester and microcystin LR were first dissolved in DMSO and then diluted 10 000-fold to achieve the desire concentration in the intracellular recording solution. Pervanadate was prepared by mixing one part of 50 mM H_2O_2 with five parts of 10 mM sodium orthovanadate in normal extracellular solution, and then incubated at room temperature for 10 min. The stock was diluted to a final concentration of 0.1 mM vanadate and 0.1 mM H_2O_2. Catalase (500 units ml^{-1}) (Boehringer Mannheim) was added right before use (Pumiglia *et al.* 1992). Pervanadate, FK506 and Hb were diluted to the desired concentration in extracellular solution. Methylene Blue was dissolved in water at 50 mg ml^{-1} and then diluted to the desired concentration in extracellular solution. These reagents were added to the bath for 15 min before applying NO or 8-Br-cGMP. ODQ (100 mM) in DMSO was diluted to 10 μM in extracellular solution before use and the cells incubated in this reagent for 1 h prior to the application of NO donors.

Statistical analysis

Statistical analysis was performed by comparing current levels (at a given time and voltage) before and after adding the different reagents with the control condition (no treatment group) by using one-way analysis of variance (ANOVA). If overall significance was found, then pairwise multiple comparisons (Dunnett's method) were performed using the SigmaStat package. Statistically significant difference was established as a P value < 0.05. The groups were defined as follows: (1) control Kv3.2 currents (time 0 and 8 min) and cGMP-treated cells (with and without biochemical manipulations); (2) control Kv3.2 and NO donor-treated Kv3.2 CHO cells (including different treatments); (3) control Kv3.1 CHO cells and cGMP (under different conditions); (4) control Kv3.1 and

D-NONOate (under different conditions); (5) control Kv3.2 and cAMP (under different conditions); and (6) control Kv3.2 cells recorded with buffer in low resistance electrodes (time 0 and 25 min) and PP2A in low resistance electrodes, PP1 in low resistance electrodes, and PP2A in high resistance electrodes. Values are expressed throughout the text as percentage of inhibition and given as means ± standard deviation (S.D.).

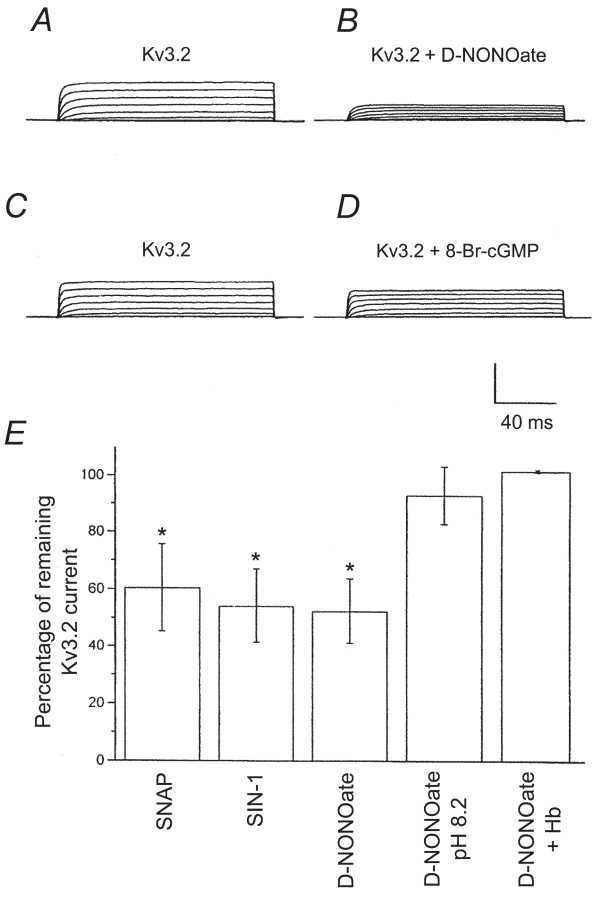

Figure 1. Effect of NO and cGMP on Kv3.2 channels expressed in CHO cells

A, Kv3.2 currents elicited in response to voltage clamp steps from −50 to +40 mV delivered every 15 s from a holding potential of −90 mV in 10 mV increments. *B*, currents in the same cell as in *A*, 8 min after bath application of 100 μM of the NO donor D-NONOate. *C*, currents from another Kv3.2-expressing CHO cell utilizing the same protocol as in *A*. *D*, same cell as in *C*, 8 min after extracellular application of 8-Br-cGMP. Vertical calibration: *A* and *B*, 1800 pA; *C* and *D*, 2000 pA. *E*, bar plots of the effect of three different NO donors on Kv3.2 currents (SNAP, SIN1 and D-NONOate) and the lack of effect of D-NONOate when applied at pH 8.2 or at pH 7.2 in the presence of 100 μM haemoglobin (Hb). Shown are means of *n* experiments as indicated in the text. Data are expressed as a percentage of the current before drug application at +20 mV. Error bars represent the S.D. *Signifcantly different from control ($P < 0.05$).

RESULTS

NO inhibits Kv3.2 channels heterologously expressed in CHO cells

CHO cells stably transfected with a Kv3.2 cDNA expressed voltage-dependent K^+ currents whose properties were similar to those of the currents observed in *Xenopus* oocytes injected with Kv3.2 cRNAs (Moreno *et al.* 1995). These currents were first observed when the membrane potential was depolarized beyond −10 mV and were of the delayed-rectifier type (Fig. 1*A*). Local, extracellular application of 100 μM of freshly prepared diethylamine NONOate (D-NONOate), a NO donor substance (Morley & Keefer 1993), at pH 7.3, resulted in reversible inhibition of the Kv3.2 currents (Fig. 1*B*). The effects of D-NONOate were very rapid, starting 5 s following application of the reagent, and reached maximal values in 5–8 min (45.8 ± 11.3 % inhibition at +20 mV, $n = 15$).

The following experiments indicate that these effects were the result of the generation of NO and not of a direct block of the channel produced by the NO donor.

D-NONOate releases NO in a pH-dependent manner (Morley & Keefer 1993). The addition of this reagent at pH 8.2 produced no effect on Kv3.2 currents (6.69 ± 10.13% inhibition at +20 mV, $n = 4$; not significantly different from control; Fig. 1*E*).

Two additional NO donor substances (with structures differing from that of D-NONOate), 3-morpholino-sydnonimine (SIN-1) and *S*-nitroso-*N*- acetylpenicillamine (SNAP) at the same concentration (100 μM) produced very similar effects (49.00 ± 17.58% ($n = 4$) and 42.9 ± 9.62% ($n = 4$) inhibition at + 20 mV; see Fig. 1*E*).

The actions of D-NONOate on Kv3.2 currents were prevented by the extracellular application of 100 μM rabbit haemoglobin (Hb) (−2.4 ± 3% inhibition at +20 mV, $n = 4$; Fig. 1*E*), which is known to bind NO with high affinity, rendering it inactive (Martin *et al.* 1985).

The inhibition of Kv3.2 channels by NO involves the cGMP signalling pathway

NO can modify channels by directly oxidizing channel proteins (Kurenny *et al.* 1994; Bolotina *et al.* 1994; Li *et al.* 1998) or indirectly through the activation of cyclic GMP-dependent protein kinases (PKG) (Robertson *et al.* 1993). To determine whether the effect of NO on Kv3.2 channels is due to activation of the cGMP signalling pathway or results from oxidation of the channel protein or a functionally associated protein, we tested the effects of permeant analogues of cGMP on Kv3.2 currents in CHO cells.

Bath application of a membrane permeant analogue of cGMP, 8-bromo-cGMP (8-Br-cGMP; 1 mM), to transfected Kv3.2 CHO cells also inhibited Kv3.2 currents reversibly, although the effects were smaller in magnitude than

those of NO donors (inhibition of 30.49 ± 14.8% from control at +20 mV, $n = 13$; Figs 1*C* and *D* and 2). The differences in magnitude of the effects of the cGMP analogue as compared to those of NO donors are probably the result of the relatively low permeability of the plasma membrane to cyclic nucleotide analogues (Forn & Greengard, 1978). Moreover, simultaneous application of 8-Br-cGMP and D-NONOate did not produce more inhibition than that produced by D-NONOate alone, suggesting a convergent pathway for both reagents (data not shown).

An additional result supporting the hypothesis that the effects of NO are mediated by activation of the cGMP cascade was obtained in experiments in which Kv3.2-transfected CHO cells were preincubated with 100 μM Methylene Blue, an inhibitor of guanylyl cyclase (GC) shown to decrease intracellular levels of cGMP (Bolotina *et al.* 1994). Methylene Blue prevented the effects of D-NONOate on Kv3.2 currents (0.075 ± 2.09% inhibition at +20 mV, $n = 4$, Fig. 2), but did not interfere with the effect produced by 8-Br-cGMP (24.72 ± 9.34% inhibition at +20 mV, $n = 5$; Fig. 2). Preincubation of cells with 10 μM ODQ, an inhibitor of the NO-sensitive GC, which does not affect particulate GC or adenylyl cyclase (Garthwaite *et al.* 1995) also prevented the effects of D-NONOate (2.72 ± 5.16% inhibition at +20 mV, $n = 5$; Fig. 2).

The effects of elevation of intracellular levels of cGMP on Kv3.2 currents are produced by the activation of cGMP-dependent protein kinase

The increase in the intracellular concentration of cGMP produced by NO or 8-Br-cGMP could result in blockade of Kv3.2 channels by direct binding to the channel protein or by activating cGMP-dependent protein kinases (PKG). To discriminate between these two possible mechanisms we tested the effects of KT5283, a specific inhibitor of

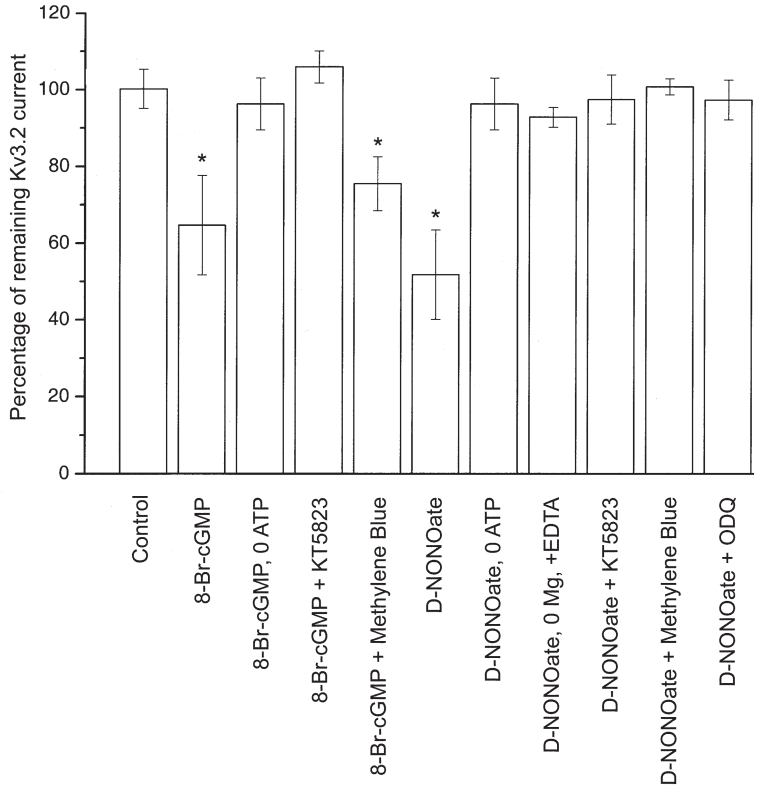

Figure 2. NO and cGMP effects on Kv3.2 channels require protein kinase activity

Bar plots comparing the values of Kv3.2 currents at +20 mV after 8 min of recording in control solutions (relative to initial values) with the values 8 min following the application of extracellular 8-Br-cGMP in normal intracellular solution, in intracellular solution without ATP, in the presence of the PKG inhibitor KT5823 at 0.3 μM, or in the presence of the guanylyl cyclase inhibitor Methylene Blue at 100 μM. Also shown are the effects of D-NONOate on Kv3.2 currents in cells with normal intracellular solution, in cells in which the intracellular solution had no ATP or 15 mM EDTA and 0 Mg^{2+}, or when the NO donor was applied in the presence of 0.3 μM KT5823, in the presence of 100 μM Methylene Blue or in the presence of 10 μM of the NO-sensitive guanylyl cyclase inhibitor ODQ. Shown are means of *n* experiments as indicated in the text. Data are expressed as a percentage of the current before drug application. Error bars represent S.D. *Signifcantly different from control ($P < 0.05$).

PKG (Kase *et al.* 1987), on the modulation of Kv3.2 currents by D-NONOate and 8-Br-cGMP. Preincubation of Kv3.2-expressing CHO cells with 0.3 μM KT5283 for 15 min prevented the effects of D-NONOate and 8-Br-cGMP (1.75 $\pm$ 7.59% ($n = 4$) and $-5.20 \pm 4.66\%$ ($n = 4$) inhibition at +20 mV; Fig. 2). In addition, the effects of D-NONOate and 8-Br-cGMP were prevented when the pipette solution contained high EDTA concentration (15 mM) and no Mg^{2+} or no ATP (7.20 $\pm$ 2.65% ($n = 3$) and 3.75 $\pm$ 6.76% ($n = 4$) inhibition at + 20 mV; Fig. 2), consistent with the involvement of a phosphate transfer activity.

The inhibition of Kv3.2 channels by activation of PKG is not produced by phosphorylation of the channel protein at the unique PKG consensus phosphorylation site

Kv3.2 proteins have two consecutive serines present in a site matching the consensus sequence for phosphorylation by PKA and PKG (Kenelly & Krebs, 1991; Fig. 3*A*). It has been shown that this site is phosphorylated by PKA, and that this phosphorylation inhibits Kv3.2 channels (Moreno *et al.* 1995). The Kv3.2a isoform does not have any other putative PKA or PKG phosphorylation sites. In order to ascertain whether the modulation of Kv3.2

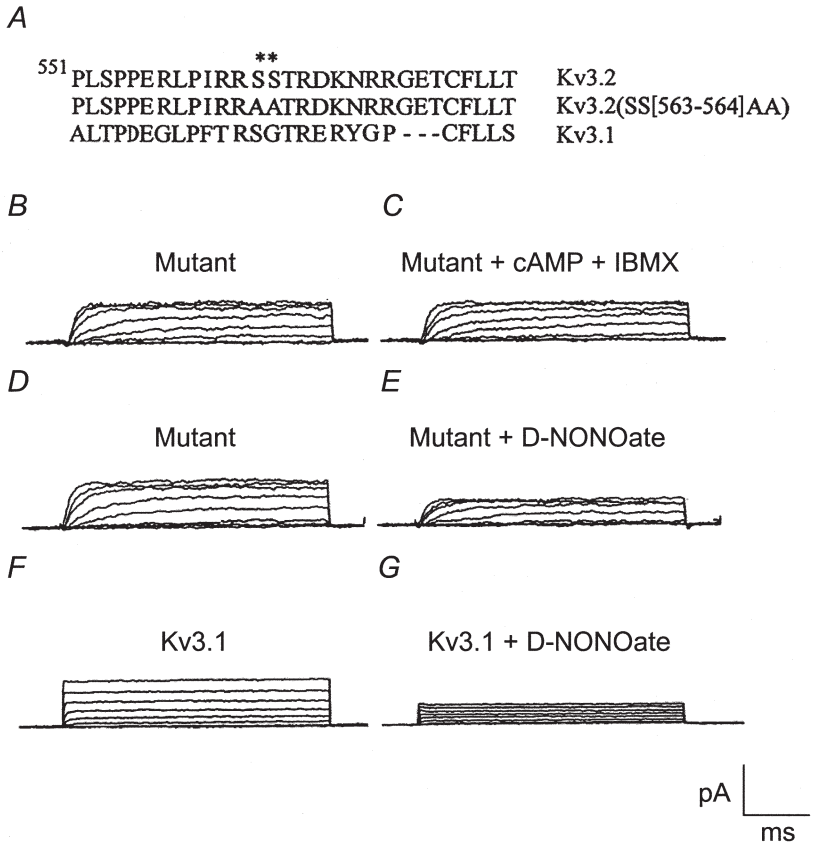

Figure 3. Modulation of Kv3.2 channels by the NO–cGMP cascade is not produced by phosphorylation of channel proteins at the unique PKA–PKG site

A, amino acid sequence comparison of the region of Kv3.2 containing the PKA–PKG phosphorylation site and the equivalent region of Kv3.1 (which lacks such a motif). Also shown is the sequence of the Kv3.2 mutant (SS[563–56]4AA) which is cAMP insensitive (Moreno *et al.* 1995). *B*, current records from a CHO cell microinjected with Kv3.2 (SS[563–564]AA) cRNA (Mutant) utilizing the same pulse protocol as in Fig. 1*A*. *C*, records from the same cell as in *B* after 15 min superfusion with 1 mM 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) and 1 mM IBMX (notice the lack of effect of these reagents which in wild-type Kv 3.2 channels produce an inhibition of 73 $\pm$ 12%) (Moreno *et al.* 1995). *D*, currents in a different CHO cell microinjected with mutant Kv3.2 cRNA (Mutant) utilizing the same pulse protocol as in *B*. *E*, same cell as in *D*, 8 min after the application of 100 μM D-NONOate. *F*, Kv3.1 currents in a Kv3.1-transfected CHO cell in response to depolarizing pulses as in *B*. *G*, same cell as in *F*, 8 min after bath application of 100 μM D-NONOate. Calibrations (vertical followed by horizontal): *B* and *C*, 200 pA and 20 ms; *D* and *E*, 180 pA and 20 ms; *F* and *G*, 1000 pA and 60 ms.

channels by NO and cGMP also results from phosphorylation of this site, CHO cells were microinjected with *in vitro* transcribed RNA (cRNA) from a cDNA in which the two serines in the unique consensus site for PKA–PKG phosphorylation have been replaced by alanines by site directed mutagenesis (Kv3.2 SS[563–564]AA). This mutation has been shown to abolish the modulatory effects of CPT-cAMP + IBMX (used to activate PKA) on Kv3.2 channels (Moreno *et al.* 1995; see also Fig. 3*B* and *C*). Bath application of either D-NONOate (Fig. 3*D* and *E*) or 8-Br-cGMP (not shown) produced an identical inhibition of the Kv3.2 currents of CHO cells expressing mutant Kv3.2 channels (49.18 ± 18.04%, $n = 4$ and 26.11 ± 11.5%, $n = 4$, at + 20 mV) to that in cells expressing wild-type channels, although the response to CPT-cAMP + IBMX was abolished as previously reported (Moreno *et al.* 1995). This result indicates that phosphorylation at the PKA–PKG consensus site is not involved in the effects of PKG activation on Kv3.2 channels. Given these observations, the effects of NO donors and 8-Br-cGMP were tested on the channels expressed by Kv3.1 subunits in CHO cells. Kv3.1 proteins are homologues of Kv3.2 and share significant sequence similarity, but lack the PKA–PKG consensus site (see Fig. 3*A*) and are not affected by the activation of PKA (Moreno *et al.* 1995). Superfusion of either D-NONOate (Fig. 3*F* and *G*) or 8-Br-cGMP (not shown) produced similar inhibition on Kv3.1 currents (53.5 ± 13%, $n = 4$ and 19.7 ± 8%, $n = 5$, at +20 mV, respectively) to those observed on Kv3.2 channels.

Modulation of Kv3 channels by PKG is mediated by a protein phosphatase

Since Kv3.1b and Kv3.2a proteins do not have additional serines or threonines in consensus sites for PKG phosphorylation, the previous result suggests at least two possible scenarios to explain the inhibition of the channel following activation of PKG. First, that PKG phosphorylates the channel at a site which does not conform to the known consensus phosphorylation sequence. Second that PKG phosphorylates an intermediary protein, which in turn affects channel function. The following set of experiments strongly supports the latter hypothesis and suggests that the intermediary protein is a protein phosphatase.

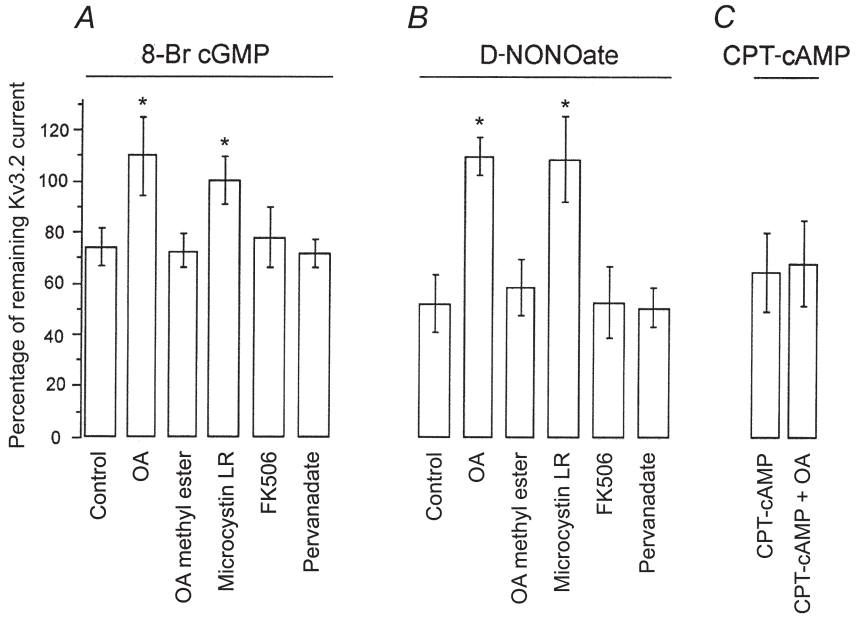

Figure 4. Specific phosphatases mediate the modulation of Kv3.2 channels by the NO–cGMP cascade

Bar plots comparing the effects of 8-Br-cGMP (*A*), or D-NONOate (*B*) on Kv3.2-expressing CHO cells recorded under control conditions or when the pipette solution contained 10 nM of the serine–threonine phosphatase inhibitor okadaic acid (OA) or its inactive form, okadaic acid methyl ester at 10 μM; or when the 8-Br-cGMP and D-NONOate were applied in the presence of 25 nM of the serine–threonine phosphatase inhibitor microcystin LR, or in the presence of 5 μM of the phosphatase inhibitor FK506 or in cells pretreated for 25 min with 0.1 mM of the tyrosine phosphatase inhibitor pervanadate. Also shown (*C*) are the effects of the permeant cAMP analogue CPT-cAMP, and CPT-cAMP in the presence of 10 nM okadaic acid (OA). Shown are means of n experiments as indicated in the text. Data are expressed as a percentage of the current before drug application at +20 mV. Error bars represent S.D. *Signifcantly different from control ($P < 0.05$).

The effects of D-NONOate and 8-Br-cGMP on Kv3.2 currents were blocked entirely (7.23 ± 15.41% ($n = 6$) and 6.02 ± 6.93% ($n = 6$) stimulation, respectively, at +20 mV; Fig. 4*A* and *B*) by the presence of 10 nM of the phosphatase inhibitor okadaic acid (OA) in the recording pipette. In fact, in all experiments, addition of okadaic acid produced a small but reproducible current increase, suggesting that basal phosphatase activity produces a small inhibition of channel activity. Okadaic acid methyl ester, an okadaic acid analogue with no apparent activity as protein phosphatase inhibitor (Nishiwaki, 1990), at concentrations of up to 100 μM, did not affect the modulation of Kv3.2 channels by D-NONOate or 8-Br-cGMP (Fig. 4*A* and *B*). We also found significant inhibition of the effects of D-NONOate and 8-Br-cGMP on Kv3.2 currents by the addition of 25 nM microcystin LR (MC) to the pipette ($n = 4$) (Fig. 4*A* and *B*). At this concentration, microcystin blocks different protein phosphatases including PP1, PP2A and PPX. On the other hand, at the concentrations of okadaic acid found to be effective in preventing the inhibition of Kv3.2 channels by NO and cGMP, this reagent blocks PP2A, and the related PPX and PP5 (Bialojan & Takai, 1988; Brewis *et al.* 1993; Skinner *et al.* 1997). We also investigated whether other protein phosphatase inhibitors were capable of preventing the modulation of Kv3.2 channels by NO and cGMP. Blockage of the FKBP class of PPIases with up to 5 μM FK506 for 4 h (Liu *et al.* 1991) or a 1 h treatment of Kv3.2-expressing CHO cells with 0.1 mM pervanadate, a non-specific tyrosine phosphatase inhibitor (Pumiglia *et al.* 1992), did not produce any significant reduction of the effects of NO or cGMP on Kv3.2 currents (Fig. 4*A* and *B*). The effects of D-NONOate and 8-Br-cGMP on Kv3.1 currents were also blocked (−12.02 ± 7% ($n = 4$) and 7.02 ± 4% ($n = 4$) inhibition, respectively, at + 20 mV) by 10 nM okadaic acid. However, okadaic acid had no effect on the inhibition of Kv3.2 currents by CPT-cAMP (Fig. 4*C*).

These results indicate that the inhibition of Kv3.2 and Kv3.1 channels by PKG is mediated by the action of specific phosphatases of the PP2A type. To test this hypothesis directly, we examined the effects of the catalytic subunits of PP1 and PP2A when applied intracellularly (Fig. 5). For this purpose, the catalytic domain of PP1 or PP2A in a glycerol-containing buffer (glycerol was used as a vehicle to store enzyme stocks) or glycerol-containing buffer alone was added to the intracellular recording solution of low resistance patch pipettes (see Methods). When PP2A was present in the

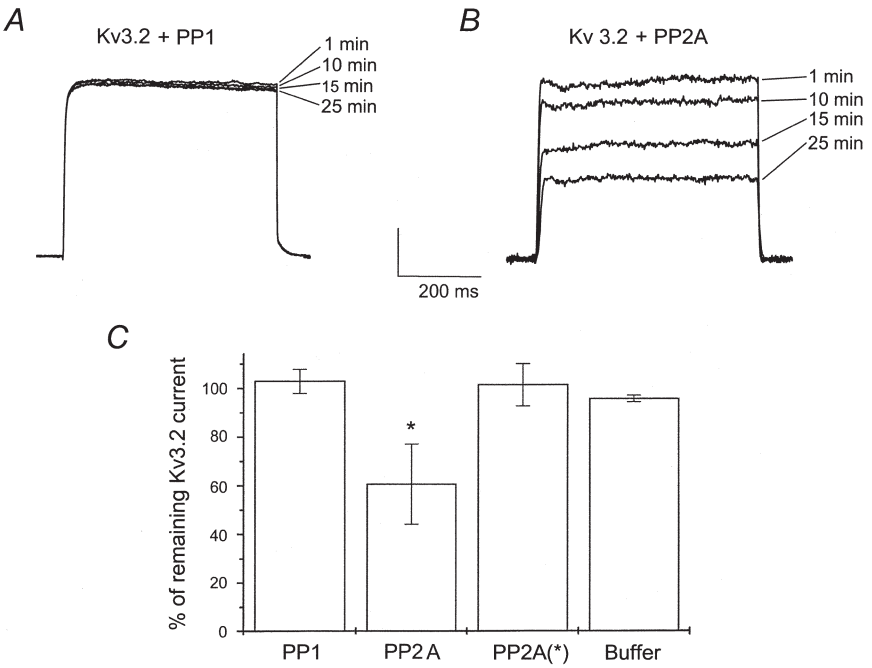

Figure 5. Protein phosphatase 2A but not protein phosphatase 1 inhibits Kv3.2 currents

Currents recorded as a function of time during a depolarization to +30 mV from a CHO cell expressing Kv3.2 channels utilizing a low resistance pipette containing the catalytic subunit of PP1 (*A*) or the catalytic subunit of PP2A (*B*). *C*, bar plots comparing the currents recorded from CHO cells expressing Kv3.2 channels with low resistance pipettes containing the catalytic subunit of PP1 or the catalytic domain of PP2A or buffer or the catalytic subunit of PP2A in high resistance pipettes (PP2A(*)). Shown are the values of the current remaining after 25 min of recording (as a percentage of the current at time zero, immediately after whole-cell access was established) during a depolarizing pulse to +30 mV ($n = 5$ for all groups except PP2A(*) for which $n = 3$). Error bars are S.D. *Significantly different from all the other experimental conditions. Vertical calibration: *A*, 400 pA; *B*, 110 pA.

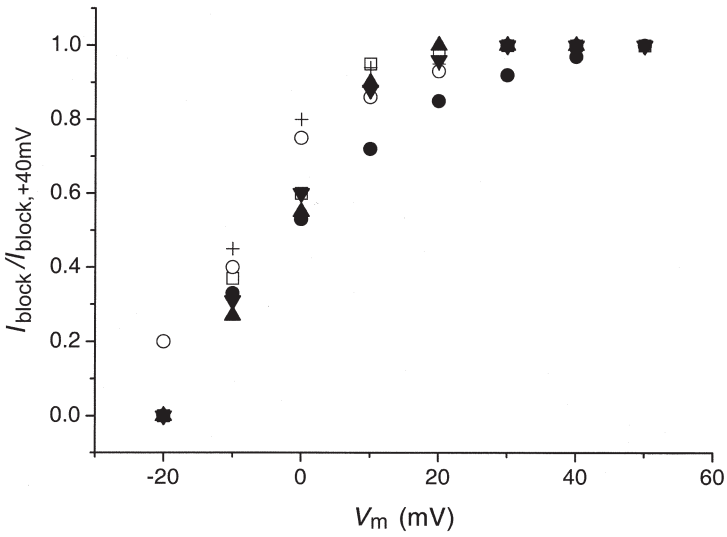

Figure 6. The degree of inhibition of Kv3.2 channels by NO and cGMP depends on the membrane potential

Plot of the relative block of Kv3.2 currents produced in six representative cells by D-NONOate (open symbols and +) and 8-Br-cGMP (filled symbols) as a function of the membrane potential during the pulse. Plotted is the ratio of current block at the indicated voltage to the block at +40 mV. All the measurements were made 8 min after application of the reagents.

pipette, the current decayed over time (39.9 ± 16.4% inhibition at +20 mV after 25 min of recording, $n = 5$) in CHO cells expressing Kv3.2 proteins (Fig. 5*B* and *C*). In contrast this decay was not observed in cells recorded with a similar concentration of the structurally related PP1 (Fig. 5*A* and *C*), or with buffer alone in the pipette, or if PP2A was included in a high resistance pipette (−2.96 ± 4.93% ($n = 5$), 4.23 ± 1.36% ($n = 5$) and −1.47 ± 8.7% ($n = 3$) inhibition, respectively, after 25 min of recording; Fig. 5*C*). These data demonstrate that PP2A has similar effects on Kv3.2 currents to PKG activation. Together with the results obtained with

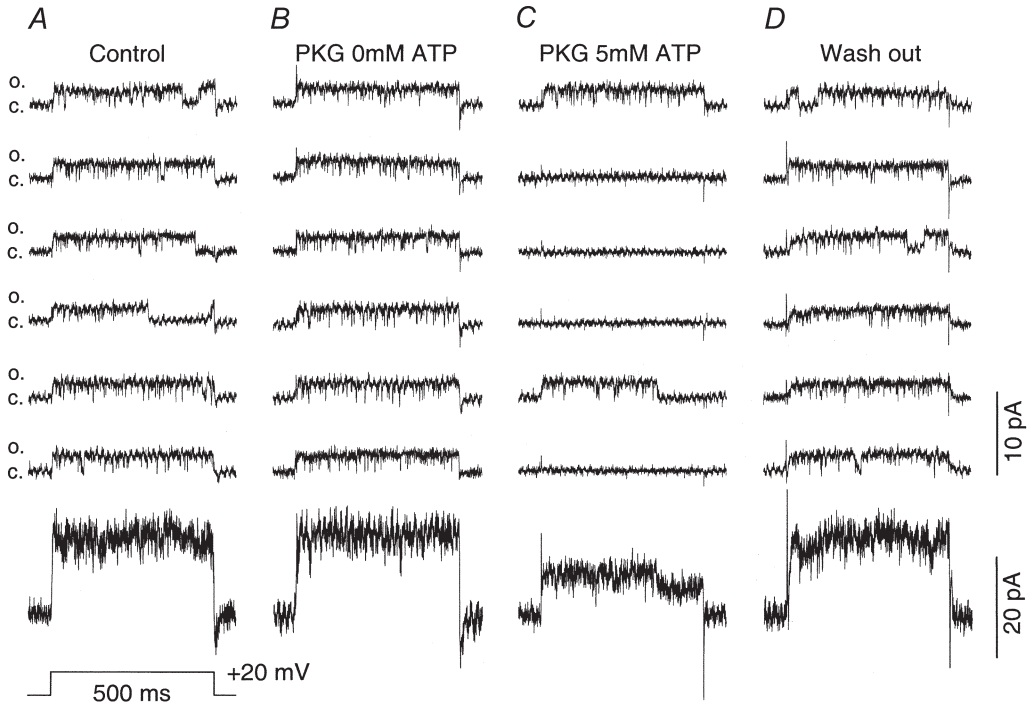

Figure 7. Inhibition of Kv3.2 channels by the catalytic subunit of PKG in excised patches

Single channels in an inside-out excised patch from a Kv3.2 stably transfected CHO cell during 500 ms depolarizations to +20 mV delivered at a rate of 1 every 10 s. *A*, representative sweeps and ensemble average from 10 consecutive sweeps recorded before application of any reagent (control); *B*, representative sweeps and ensemble average from 10 consecutive sweeps recorded during local application of the catalytic subunit of PKG (1.5 U μl^{-1}) to the cytoplasmic side of the excised patch in the absence of ATP. Note that there is no significant change compared to the control condition. *C*, representative sweeps and ensemble average from 10 consecutive sweeps recorded in the presence of PKG and 5 mM ATP. Under these conditions there is a clear reduction of channel openings. *D*, representative sweeps and ensemble average from 10 consecutive sweeps recorded 8 min after perfusion with normal bath solution (see Methods). O, open state; C, closed state.

phosphatase inhibitors, the data suggest that the phosphatase activated by PKG in CHO cells which modulates Kv3.2 currents is of the PP2A type. Nevertheless, it is still possible that CHO cells have other unknown highly OA-sensitive phosphatases.

Kv3 channels are inhibited by the NO–cGMP system to different degrees, depending on the membrane potential

D-NONOate or 8-Br-cGMP did not reduce Kv3.1 and Kv3.2 currents by the same amount at all voltages. Current block was more efficient when tested with larger depolarizing pulses, reaching a maximum at voltages above +20 mV ($n = 16$). This characteristic voltage-dependent inhibition, always larger with larger depolarizations, was observed with both D-NONOate and 8-Br-cGMP and with both Kv3.1 (not shown) and Kv3.2 channels (Fig. 6). The voltage dependence of channel inhibition shown in Fig. 6 is similar to the voltage dependence of channel activation (Rudy *et al.* 1999).

Kv3.2 channels are closely associated with the molecular elements of the NO cascade

Inhibition of Kv3.2 channels by the PKG system could also be demonstrated at the single channel level. Single channel recordings of Kv3.2-transfected CHO cells were performed in the inside-out configuration of the patch clamp technique (Hamill *et al.* 1981). Channels were recorded during large depolarizations, when the probability of channel opening is high (Fig. 7). As shown in Fig. 7*B*, local application of the catalytic subunit of PKG (1 U μl^{-1}) to the cytosolic side of the membrane in the absence of ATP did not produce any significant change in channel activity. However, the addition of ATP in the presence of the catalytic subunit resulted in a large inhibition of channel activity (Fig. 7*C*). Channel activity recovered upon removing the catalytic subunit of PKG (Fig. 7*D*), or the ATP (data not shown), and in this particular patch the same series of events were reproduced four times until the patch was lost. These experiments were repeated with similar results in several patches pulled from three independent cells. These results are consistent with the notion that both the phosphatase activated by PKG and the kinase system that phosphorylates the target site of that phophatase are present and retain their functionality in the area of membrane and/or cytosolic elements present in the cell-free patch.

DISCUSSION

The experiments described here have unravelled a cascade of molecular events involved in the modulation of Kv3.1 and Kv3.2 channels by the signalling gas NO via a cGMP-dependent signalling pathway. Inhibition of the channels by NO requires both phosphorylation and dephosphorylation reactions. To investigate the steps involved in this process, we studied the effects of substances capable of interfering with or mimicking at different levels the NO–cGMP-dependent pathway: (1) NO donors, (2) cGMP production, (3) phosphate donors, (4) PKG activity, and (5) phosphatase activity. We found that elevation of intracellular concentration of cGMP results in a voltage-dependent inhibition of Kv3.1 and Kv3.2 channels.

Several types of K^+ channels have been shown to be modulated by the cAMP pathway, but much less is known about targets of the cGMP system. Nevertheless, previous studies have shown NO–cGMP-dependent and/or independent stimulation of several channels, including Ca^{2+}-activated, ATP-dependent and voltage-gated K^+ channels (White *et al.* 1993; Bolotina *et al.* 1994; Ahern *et al.* 1999). The present study identified new targets of these signalling systems and showed that activation of the cGMP pathway can also produce voltage-gated K^+ channel inhibition.

Signalling pathway involved in the modulation of Kv3 channels by the NO–cGMP signalling system

Experimental evidence is presented consistent with the notion of a signalling pathway in which NO increases intracellular cGMP that in turn activates PKG. The activation of PKG increases the activity of a phosphatase which then produces inhibition of channel activity.

NO interacts with diverse targets exerting effects via redox mechanisms (Stamler, 1994). One of the best-characterized targets of NO is the soluble form of guanylyl cyclase (sGC), which is activated upon interaction with NO (Bredt & Snyder, 1994). The activation of sGC by NO results in large increases in intracellular cGMP. Downstream effects can follow the elevation of cGMP levels. The observed inhibition of Kv3.1 and Kv3.2 currents by NO is mimicked by the addition to the extracellular medium of membrane-permeant cGMP analogues and is prevented by inhibitors of sGC or PKG. These results demonstrate that channel inhibition is the result of the NO-mediated increases in cGMP and is not due to the direct interaction of NO (or other oxidizing derivatives) with the channel protein, as proposed for the effects of this molecule on Na^+ and Ca^{2+} channels, Ca^{2+}-activated K^+ and cyclic nucleotide-gated channels, as well as NMDA and ryanodine receptors (Lipton *et al.* 1993; Bolotina *et al.* 1994; Broillet & Firenstein 1996; Li *et al.* 1998; Xu *et al.* 1998).

Although PKG is involved in the cascade of events by which NO inhibits Kv3.1 and Kv3.2 channels, the results suggest that this inhibition is not due to phosphorylation of the channel proteins. Kv3.1 channels lack a consensus sequence for PKG phosphorylation. Although Kv3.2 channels do have a putative PKA and PKG phosphorylation site, mutation of the serines in this site does not prevent channel inhibition by NO or cGMP. The same levels of inhibition are obtained when these serines are replaced by alanines.

We have previously shown that the PKA and PKG phosphorylation site in Kv3.2 is phosphorylated by PKA thereby producing channel inhibition (Moreno *et al.* 1995). Since PKG activation produces identical effects on Kv3.2 channels when phosphorylation of this site is prevented by mutagenesis, it is most likely that PKG is not capable of phosphorylating this site. The target of the PKG in our system appears to be a protein phosphatase. Based on the pharmacological profile, in particular the sensitivity to low concentrations of okadaic acid and the effects produced by the intracellular dialysis of the catalytic domain of PP2A but not PP1, we propose that the phosphatase activity induced by the NO–cGMP cascade which inhibits channel activity in CHO cells belongs to the family of serine–threonine phosphatases, most likely PP2A or a PP2A-related enzyme (such as PPX or PP5). However, whether the activation of this enzyme results from direct phosphorylation by PKG of the catalytic subunit and/or one of its regulatory subunits, or from the phosphorylation of an associated protein which in turn modulates the activity of the phosphatase remains to be clarified. This is of considerable interest because few direct targets of PKG are known. It has been shown that one target of PKG is the phosphatase inhibitor DARPP-32, a dopamine- and cAMP-regulated phosphoprotein that in substantia nigra can be phosphorylated by PKG in response to NO (Tsou *et al.* 1993).

The molecular cascade by which NO inhibits Kv3.1 and Kv3.2 channels is similar to that proposed for the activation of Ca^{2+}-activated K^+ channels by natriuretic peptide (White *et al.* 1993). In this study the effects are mediated by PKG and also involve an okadaic acid-sensitive protein phosphatase. In none of the cases, including the present, was it possible to differentiate between the phosphatases known to be highly sensitive to okadaic acid (PP2A, PPX and PP5). Nevertheless, in CHO cells, tracheal smooth muscle and mesangial cells, it has been suggested that PP2A is essential for the PKG modulation of Ca^{2+}-activated K^+ channels (Zhou *et al.* 1996).

The mechanism by which activation of the phosphatase results in channel inhibition also remains to be clarified. One possibility is that the channel protein is dephosphorylated at (a) critical site(s), which needs to be phosphorylated in order for the channel to be activated by membrane depolarization. This possibility is consistent with our previous findings that alkaline phosphatase inhibits the currents expressed by Kv3 channels in *Xenopus* oocytes and CHO cells (Vega-Saenz de Miera *et al.* 1995). The idea that certain site(s) in Kv3 channels have to be phosphorylated for proper channel activation is also consistent with the observation that the addition of phosphatase inhibitors not only prevented the NO–cGMP-dependent inhibition of channel activity but also consistently produced stimulation of basal channel activity (Fig. 4). However, biochemical experiments are still required to demonstrate that Kv3 channel proteins are dephosphorylated by protein phosphatases. At this stage the possibility of additional intermediaries between phosphatase activation and channel inhibition cannot be eliminated.

The reversibility of the effects of NO in whole-cell or patch recordings suggests that the elements of the signalling pathway described here, and in particular the kinase which reactivates channel function once the phosphatase activity is suppressed, are quite active. Also, in the basal state, this kinase must be tonically active, so that the channels are functional. The identity of such a kinase also remains to be identified.

Voltage dependence of the inhibition of Kv3.1 and Kv3.2 channels by the NO–cGMP system

We have shown that the inhibition of Kv3.1 and Kv3.2 currents by NO donors and 8-Br-cGMP is larger as the membrane depolarization increases. There are at least two mechanisms that could explain this result. First, it is possible that the membrane potential is affecting some element(s) of the signalling cascade. Since the voltage dependence of the effects of D-NONOate and 8-Br-cGMP is the same, the voltage-dependent element(s) would have to be downstream of the activation of GC. In support of this possibility, it was recently demonstrated that the cAMP-dependent phosphorylation of L-type Ca^{2+} channels in skeletal muscle cells requires membrane depolarization, perhaps via a voltage-dependent recruitment to the membrane of the proper A-kinase-anchoring proteins (AKAP) (Johnson *et al.* 1994). However, to explain our results the kinetics of the change(s) induced by depolarization on the signalling molecules would have to be very fast.

The second possibility is that the signals initiated by activation of PKG are affecting preferentially channel states that operate during large depolarizations. Single channel recordings from patches of CHO cells expressing Kv3.2 (Fig. 7*A*) show brief as well as prolonged transitions to a closed sate. A kinetic scheme such as the one shown below could account for these data:

$$C_2 \rightleftharpoons C_1 \rightleftharpoons O$$

During a depolarization the open channel (O) can close to either the C_1 or C_2 closed states, accounting for the observed brief and long closings. This kinetics does not seem to be affected by the addition of PKG in the presence of ATP (Fig. 7*C*). Instead, the probability of obtaining sweeps where the channel opens at all is drastically reduced. PKG-dependent dephosphorylation of the channel protein could decrease the probability of opening if the transition between the two closed states, or between the last closed state (C_1) and the open state depended on the phosphorylation of (a) critical site(s) in the channel protein.

Physiological significance

Kv3.1 and Kv3.2 proteins are expressed in the soma and axons of specific neuronal populations in the mammalian CNS (see Introduction). There is overlap between neuronal populations expressing Kv3.2 and Kv3.1 channels and NO both in somata and fibres. For example, both NOS and Kv3.2 and/or Kv3.1 proteins are expressed in somatostatin-containing neurons in the hippocampus; in retinoceptive layers of the superior colliculus; in thalamic and reticular thalamic neurons; in the olfactory bulb; in the globus pallidus; and in cerebellar granule cells (see Introduction). However, more detailed immunohistochemical studies are required to confirm that NOSs, the other elements of the signalling pathway, and the channel proteins are co-expressed in the same cells and in the appropriate locations to interact as they do in the heterologous expression system studied here.

NO is not the only signal that can trigger a rise in cGMP. Natriuretic peptide (White *et al.* 1993), vasoactive intestinal peptide (VIP), bradykinin, ATP, angiotensin, endothelin, muscarinic and nicotinic receptors, and the labile gas CO (Verma *et al.* 1993) can all elevate the intracellular concentration of cGMP. Interestingly the kinetics of cGMP responses elicited by NO and muscarinic receptor activation have similar rising phase but different falling phase kinetics (Trivedi & Kramer 1998). Activation of voltage-gated Ca^{2+} channels has also been reported to produce elevation of cGMP.

The molecular elements involved in the NO–cGMP modulation of Kv3.2 or Kv3.1 channels include at minimum GC, PKG and a serine–threonine phosphatase. These molecules have been found in several of the brain areas expressing Kv3.1 and Kv3.2 channels, although more refined anatomical studies are required to determine if proper colocalization of the proteins exist in any given neuronal system. Areas that are particularly relevant for such studies include the thalamus, where NO alters the oscillatory activity of thalamic relay neurons (Pape & Mager 1992), or in the reticular thalamic nucleus. Another area of interest for future colocalization and electrophysiological studies is the superior colliculus, which also expresses GC and PP2A mRNAs (Abe *et al.* 1994).

Taken together, these results demonstrate that heterologously expressed Kv3.1 and Kv3.2 channels can be inhibited by a series of molecular events initiated by the induction of the NO–cGMP cascade, which involve the activation of a serine–threonine phosphatase. Furthermore, since the different elements involved in this cascade appear to be localized in areas expressing Kv3.1 and Kv3.2 proteins, similar modulation of channel activity may take place *in vivo*.

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