Eur Biophys J (2005) 34: 217–229
DOI 10.1007/s00249-004-0444-x

ARTICLE

Mauro Toselli · Vanni Taglietti

L-type calcium channel gating is modulated by bradykinin with a PKC-dependent mechanism in NG108-15 cells

Received: 28 June 2004 / Revised: 14 September 2004 / Accepted: 16 September 2004 / Published online: 5 November 2004

Abstract Bradykinin (BK) excites dorsal root ganglion cells, leading to the sensation of pain. The actions of BK are thought to be mediated by heterotrimeric G protein-regulated pathways. Indeed there is strong evidence that in different cell types BK is involved in phosphoinositide breakdown following activation of $G_{q/11}$. In the present study we show that the Ca^{2+} current flowing through L-type voltage-gated Ca^{2+} channels in NG108-15 cells (differentiated in vitro to acquire a neuronal phenotype), measured using the whole-cell patch clamp configuration, is reversibly inhibited by BK in a voltage-independent fashion, suggesting a cascade process where a second messenger system is involved. This inhibitory action of BK is mimicked by the application of 1,2-oleoyl-acetyl glycerol (OAG), an analog of diacylglycerol that activates PKC. Interestingly, OAG occluded the effects of BK and both effects were blocked by selective PKC inhibitors. The down modulation of single L-type Ca^{2+} channels by BK and OAG was also investigated in cell-attached patches. Our results indicate that the inhibitory action of BK involves activation of PKC and mainly shows up in a significant reduction of the probability of channel opening, caused by an increase and clustering of null sweeps in response to BK.

Keywords Calcium channel · Bradykinin · Protein kinase C · NG108-15 · GPCR

Introduction

The activation of voltage-dependent L-type calcium channels transduces electrical stimuli into contraction in cardiac and skeletal muscle cells (Striessnig 1999; Tanabe et al. 1987), and into hormone release in neuroendocrine cells (Ashcroft et al. 1994; Barg et al. 2001). In neurons, L currents play a significant role in multiple cellular functions including neurotransmitter release (Rane et al. 1987; Evans and Pocock 1999; Sand et al. 2001), regulation of gene expression (Finkbeiner and Greenberg 1998; Dolmetsch et al. 2001), dendritic development (Schwab et al. 2001) and synaptic plasticity (Weisskopf et al. 1999; Akopian and Walsh 2002). In addition to voltage and electrical activity, several neurotransmitters, hormones and peptides activate a variety of signaling pathways to regulate the influx of Ca^{2+} through L channels. Although this modulation has been thoroughly characterized in cardiac and endocrine cells (Kamp and Hell 2000; Carbone et al. 2001), only a few studies have focused on L channel modulation in neurons.

Inhibition of neuronal L channels occurs via stimulation of G-protein-coupled receptors (GPCR) and subsequent activation of $G_{i/o}$ and/or $G_{q/11}$-mediated pathways (Ewald et al. 1989; Howe and Surmeier 1995; Wilk-Blaszczak et al. 1996). Recent studies indicate that L currents generally do not exhibit the voltage-dependent inhibition that is typical of N- or P/Q-type Ca^{2+} currents (Mathie et al. 1992; Bourinet et al. 1996; Bell et al. 2001), produced by the direct binding of $G\beta\gamma$ to the channel (Herlitze et al. 1996; Ikeda 1996). Rather, a number of voltage-independent forms of L channel inhibition have been reported, including phospholipase Cβ1 activation-dependent inhibition (mediated by $G_q\beta\gamma$) (Howe and Surmeier 1995; Hernandez-Lopez et al. 2000), $G\alpha_{q/11}$-dependent inhibition (Bannister et al. 2002), $G_{i/o}$-stimulated release of Ca^{2+} from intracellular stores (Kramer et al. 1991; Oz et al. 1998), or $G_{i/o}$-mediated activation of protein kinase C (McCullough et al. 1998). The results obtained by Boland et al. (1991) and by Haimes et al. (1992) are also consistent with the potential role of PKC in the negative regulation of neuronal L channels. The involvement of PKC in the inhibition of Ca^{2+} currents is, however, controversial (Hockberger et al. 1989; Bley and Tsien 1990).

M. Toselli (✉) · V. Taglietti
Dipartimento di Scienze Fisiologiche e Farmacologiche Cellulari e Molecolari and INFM, Universita' di Pavia, Via Forlanini 6, 27100 Pavia, Italy
E-mail: mtoselli@unipv.it

Previous studies on neuronal NG108-15 cells and other cell types have reported that the pain-producing peptide bradykinin (BK) stimulates a variety of second messenger systems, one or more of which might be involved in the regulation of L channels by BK. Indeed, BK was reported to induce an increase in phosphoinositide breakdown in NG108-15 cells (Osugi et al. 1987), in which L channel inhibition by both $G_{i/o}$ and $G_{q/11}$, following BK receptor activation, has been documented as well (Wilk-Blaszczak et al. 1996; Connor and Henderson 1997; Tosetti et al. 2003).

Based on this preliminary evidence and the availability of useful pharmacological tools, and using both the whole-cell and cell-attached configurations of the patch-clamp technique, we tested the hypothesis that BK inhibits L-type Ca^{2+} channel activity in NG108-15 cells via a mechanism that, involving the activation of PKC, causes depression of channel activity. The decrease of phosphatidyl-inositol-4,5-bisphosphate concentration as a potential mechanism of channel inhibition is also discussed.

Materials and methods

Cell culture

Rat NG108-15 neuroblastoma × glioma hybrid cells were grown in monolayers in DMEM (Dulbecco's Modified Eagle Medium), 10% heat-inactivated fetal calf serum, HAT (hypoxantine-aminopterin-thymidine) supplement, 100 μg streptomycin/ml, and 100 IU penicillin/ml in a 5% CO_2 humidified atmosphere at 37 °C. Cells were grown in plastic flasks, and plated in plastic Petri dishes for use in electrophysiological experiments. The culture medium was replaced three times per week.

To induce cell differentiation into a neuronal phenotype and Ca^{2+} channel expression (Kasai and Neher 1992), 10 μM prostaglandin E_1 (ICN Biochemicals, Italy) and 1 mM theophylline were added to the culture medium of NG108-15 cells at least five days prior to electrophysiological recordings.

Solutions

For the whole-cell recordings, the patch pipettes were filled with (mmol/l): 125 CsCl, 20 tetraethylammonium chloride, 10 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, 4 Mg-ATP, 0.1 GTP, 10 HEPES/CsOH (pH 7.4). Seals between electrodes and cells were established in a solution containing (mmol/l): 135 NaCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5.5 KCl, 10 glucose, 10 HEPES/NaOH (pH 7.4). After establishing the whole-cell configuration, cells were perfused with an external saline containing (mmoles/l): 135 NaCl, 10 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES/NaOH (pH 7.4), 1,4-aminopyridine, 10 tetraethylammonium chloride, 10^{-3} tetrodotoxin (TTX). Most differentiated NG108-15 cells expressed low-voltage-activated T-type, and high-voltage-activated L- and N-type Ca^{2+} channels (Kasai and Neher 1992; Tsunoo et al. 1986). L-type currents were isolated by pre-incubating the cells for about 10 min with 1 μM ω-conotoxin GVIA (Alomone Labs, Israel), and by focal perfusion of the tested cells with the same amount of toxin dissolved in the external recording solution to block N-type channels, and by holding the membrane potential of the cell at −40 mV to inactivate T-type Ca^{2+} currents. Under these conditions, a second application of the toxin at a concentration of 10 μM caused a negligible further decrease in the Ca^{2+} current ($6 \pm 4\%$, $n = 5$), demonstrating that 1 μM ω-conotoxin GVIA was effective in completely blocking N-type channels. For all experiments, BK was diluted into the extracellular solution to a working concentration of 100 nM. External solutions were exchanged using a fast multi-barrel delivery system positioned close to the recorded cells.

The protein kinase C (PKC) activators 1-oleyl-2-acetyl-sn-glycerol (OAG) and phorbol 12-myristate 13-acetate (PMA), (and its inactive form 4-α-PMA), the PKC inhibitors staurosporine and bisindolylmaleimide I (Bis I) (and its negative control bisindolylmaleimide V (Bis V)), the PLC inhibitor U-73122 (together with its inactive control U-73343) and the phosphoinositide 4-kinase inhibitors phenyl-arsine oxide (PAO) and wortmannin were dissolved in DMSO to make stock solutions. Immediately before use, a dilution of the required drug was prepared in intra- or extracellular saline such that the DMSO concentration never exceeded 0.1%.

For the cell-attached recordings, the pipette control solution contained (mmol/l): 100 $BaCl_2$, 10 TEA-Cl, 1 $MgCl_2$, 10 Na-HEPES, plus 1 μM ω-conotoxin GVIA, 300 nM TTX, and 5 μM Bay K 8644 (pH 7.3 with TEA-OH). Membrane potential was zeroed with a solution containing (mmol/l): 135 potassium aspartate, 1 $MgCl_2$, 10 HEPES, 5 EGTA, and 300 nM TTX (pH 7.3 with KOH). For the experiments with receptor agonists in the pipette, BK 100 nM was added to the control saline. The Ca^{2+} channel agonist (±)-Bay K 8644 was a gift from Bayer A. G. (Wuppertal, Germany). Stock solutions of (±)-Bay K 8644 (5 mM) were prepared in DMSO and stored light-protected at 4 °C. Bay K was diluted daily to a final concentration of 5 μM.

Current recordings and data analysis

Patch pipettes were made from borosilicate glass tubing (Hilgenberg GmbH, Malsfeld, Germany) and fire-polished to a final resistance of 0.5–2.0 MΩ when filled with the internal solutions used. For cell-attached recording, the pipette tips were coated with Sylgard to reduce capacitance and noise. All of the experiments were performed at room temperature (22–24 °C).

Whole-cell and single channel currents were recorded with a LIST LM/EPC7 patch-clamp amplifier (HEKA), and with an Axopatch 200A amplifier (Axon Instruments) respectively, digitized at sampling intervals of

26–100 μs using a 12 bit A/D Tecmar LabMaster Board for the whole-cell recordings or a DigiData 1200 (Axon Instruments) for the single-channel recordings. Stimulation, acquisition, and data analysis were carried out with PCLAMP (Axon Instruments Inc., Burlingame, CA, USA) and ORIGIN (Microcal Software Inc., Northampton, MA, USA) software. Fast capacitive transients were reduced on-line by analog circuitry. Residual capacitive and leak currents were removed by P/4 subtraction for whole cell recordings, whereas for single channel recordings they were removed by subtracting from each active sweep an idealized current obtained by fitting the averaged silent traces (nulls) with a multiexponential function. Currents were filtered at 3 kHz.

The amount of whole-cell current inhibition by drug application was measured as follows: currents were measured at the time that control currents peaked (T_p); modulated currents were obtained by first taking the difference at T_p between the control and residual currents following drug application, and then normalizing to the control and expressing as a percentage.

Single channel event detection was performed with the 50% threshold detection method and limited to those patches containing only one channel. Such patches were identified by the absence of overlapping unitary currents at $+30/+40$ mV, at which the probability of channel opening is relatively high, even in the presence of BK or OAG, and multiple open levels could be clearly resolved when present. Furthermore, the following algorithm was applied to estimate the likelihood of single channel activity in those patches without superimposed openings (Plummer et al. 1989): $P_2(T) = 1-(1-P_{2o})^{T/t}$, where $P_2(T)$ is the cumulative probability of observing superimposed openings due to the activity of two identical channels over the total observation time T, P_{2o} is the overall probability of finding two simultaneous openings, and t is twice the mean open time. The patches used for further analysis were those with $P_2(T) > 0.999$ despite the absence of superimposed openings during the observation time T.

More critical than those for the whole cell configuration were the criteria to exclude the presence of N-type channels, and therefore positively identify only single L-type channels in each recording. In fact it has been reported that ω-conotoxins may not completely block N-type channels in 100 mM Ba^{2+} (McDonough et al. 1996). The first criterion was to discard patches containing multiple levels of openings at $+30/+40$ mV (see before). The second one was to compare, for each recording, unitary current amplitude, P_o and arithmetic mean open time with those obtained by single N-type channel analysis from the same cell type (data not shown), and take advantage of the fact that in NG108-15 cells at 0 mV all three parameters are significantly different for the two Ca^{2+} channel subtypes (N-type: $i = 0.9$ pA, $P_o = 0.02$, $t_o = 0.4$ ms; L-type: $i = 1.3$ pA, $P_o = 0.1$, $t_o = 2$ ms).

As specified in the text, open probability (P_o) was evaluated by excluding either the first and last closures of the channel and null sweeps, or by dividing the sum of the open times by the entire step duration. The first method of calculating P_o ignores the first latency and the rate of channel inactivation and is preferred for looking at changes in P_o when the channel is actively gating. It also furnishes an estimate for P_o that is independent of the length of the pulse. The mean P_o at each potential was calculated by averaging the P_o measured from each single patch over a variable number of sweeps. To construct $P_o(V)$ curves, mean P_o values were plotted versus potential and fitted to a Boltzmann equation. To estimate the mean open and mean closed times, the single channel events were log binned into open and closed time histograms, excluding the first and the last closures and limiting the analysis to events longer than twice the dead time (360 μs) (Colquhoun and Hawkes 1995). Openings were long enough and well resolved. We used two ways to determine the mean open and mean closed times, obtaining very similar results. In one case, the two parameters were estimated by averaging the arithmetic mean of the open (t_o) and closed times (t_c) of each patch. This gave estimates for the two parameters independently of the fitting procedure and the number of exponentials used for the fit, and allowed the derivation of values at various potentials when, for practical reasons, a limited number of sweeps at each potential could be collected (Fig. 2d). In the second case, all single channel events at $+10$ mV were pooled together and plotted on linear-log coordinates to construct a single open and closed time distribution which was best fitted with either two or three exponentials using the maximum likelihood method (Sigworth and Sine 1987). In this case, the distributions were constructed from a large number of traces, either coming from many patches with a small number of traces per patch or from fewer patches with many more traces per patch. The mean t_o and mean t_c values obtained from the total fit corresponded well with those obtained using the first method at the same potential ($+10$ mV). The open times were fitted well by two exponentials, while fitting the closed times required three components.

All of the data in the text and figures to follow are given as mean ± SEM for n observations. Statistical significance (p) was calculated using the Student's paired t-test. Fitting of the prepulse/postpulse data was performed by a nonlinear regression method based on the Levenberg-Marquardt algorithm. Concerning "runs analysis", used to test null sweeps for randomness, the distribution of the number of runs was approximated by an asymptotic distribution, forming a standardized random variable, Z, with a mean of zero and variance of one. For our purposes

$$Z = -\frac{R - 2n\rho(1-\rho)}{2\sqrt{n\rho(1-\rho)}}$$

where R is the number of runs, n is the total number of trials, and r is the probability of at least one channel opening during a trial. The expected number of runs is

$2n\rho(1-\rho)$. Positive values of Z correspond to clusters of sweeps with openings (Horn et al. 1984).

Results

In NG108-15 cells, L currents are reversibly inhibited by BK (Wilk-Blaszczak et al. 1996; Connor and Henderson 1997). The inhibition causes a size reduction with no change in the activation time course, and a facilitator pre-pulse to +80 mV is unable to recover the inhibition (Tosetti et al. 2003). Furthermore, both the onset and the recovery from BK-mediated inhibition are slow (about 27 and 230 s respectively), suggesting the involvement of a multi-step second messenger cascade rather than a direct membrane-delimited pathway. Wilk-Blaszczak et al. (1996) suggested that this inhibitory mechanism should involve activation of the effector enzyme phospholipase C-β (PLC-β). In the canonical signalling pathway, PLC-β cleaves phosphatidyl-inositol-4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 triggers Ca^{2+} release from intracellular stores, whereas DAG activates PKC. We used several pharmacological tools to further characterize the pathway responsible for the slow BK inhibition of L current.

Effects of activators and inhibitors of PKC on L current modulation

As illustrated in Fig. 1a, application of 5 μM 1,2-oleoylacetyl-glycerol (OAG), a DAG analogue that activates PKC (Nishizuka 1984), caused reversible inhibition of the L current. The average percentage of L current inhibition at +10 mV is 31.9 ± 5.0 (n = 18). In Fig. 1b the average L current-voltage relationships obtained in control saline and in the presence of OAG are also compared. The inhibition is similar to that observed with BK (Tosetti et al. 2003): it consists in a current size reduction with no evident change in the activation and inactivation time courses and a facilitator pre-pulse to +80 mV is unable to recover the inhibition, as shown by the sample tracings of Fig. 1c: the depolarizing pre-pulse caused a negligible recovery of current from OAG-induced inhibition (on average 2.8 ± 3.2%, n = 10). Furthermore, as shown in the representative time-plot of inhibition of Fig. 1d, the onset of OAG-mediated inhibition is relatively slow and similar to that obtained with BK (on average 26.2 ± 4.6 s, n = 4); the recovery from OAG-mediated inhibition (on average 121.5 ± 13.0 s, n = 4), although faster than that obtained with BK (~220 s), is still very slow, when compared with that observed for the membrane-delimited and voltage-dependent opioid-mediated inhibition of the N-type Ca^{2+} current (< 4 s, Toselli et al. 1997).

For comparison, another activator of PKC, phorbol 12-myristate 13-acetate (PMA) (Nishizuka 1984), was also applied. PMA caused reversible inhibition of the L current, with features similar to those produced by OAG and BK. The percentage average inhibition of L current at +10 mV following the application of PMA (1 μM) is 28.9 ± 3.3% (n = 11), as summarized in the histograms of Fig. 1g. By contrast, the non-biologically-active phorbol 4-α-PMA (1 μM), used as negative control, did not produce any significant inhibition of the L current (3.2 ± 2.3%, n = 3).

In order to test whether the inhibitory effect of OAG (and PMA) on the L current is really mediated by PKC activation or independent of its effect as activator of PKC (see Hockberger et al. 1989), we tested whether OAG could still depress the L current after inhibition of PKC in another set of experiments. The selective PKC inhibitor Bis I (Toullec et al. 1991; Cho et al. 2001) was added to the patch pipette solution at a concentration of 0.5 μM, and the cells were held for about 10 min after rupture of the patch to allow the inhibitor to diffuse into the cytoplasm. In the presence of Bis I, the average block of the L current produced by OAG (5 μM) was 4.6 ± 1.7% (n = 8). Interestingly, with the PKC blocker inside the cells, the inhibitory effect of BK (100 nM) was also strongly reduced, with an average L current block of 4.1 ± 1.6% (n = 8). By contrast, in the presence of Bis V, used as negative control (Davis et al. 1992), the percentage of L current inhibition by OAG was not significantly reduced (32.8 ± 3.7%, n = 3). Also, the potent kinase inhibitor staurosporine (Hidaka et al. 1984), when added to the patch pipette solution at a concentration of 100 nM, caused a significant reduction in the L current block produced both by OAG (5 μM) (3.9 ± 1.5%, n = 14) and by BK (100 nM) (4.8 ± 1.5%, n = 13). Therefore, following PKC inhibition, the efficacy of OAG and BK to reduce the L current underwent a net decrease of about 88%, as summarized in the histograms of Fig. 1g, ($p < 0.05$).

To further test whether OAG and BK reduced the L current through a common mechanism, in a set of seven experiments each cell was first focally perfused with 5 μM OAG alone, and then with BK (100 nM) together with OAG (sample traces in Fig. 1e). Indeed, during simultaneous application of the two drugs the amount of current inhibition did not increase significantly in comparison with that caused by OAG alone (on average, 35.1 ± 3.9% and 33.4 ± 4.4% respectively), suggesting that a converging mechanism mediates the down modulation of the L current caused by the activation of BK receptors and PKC.

Since BK receptors have been shown to be strongly linked to the $G\alpha_q$/PLC pathway in many cell types (Lee and Rhee 1995), we asked whether BK uses the PLC pathway to inhibit L currents in this case. We tested this hypothesis by using U-73122 and U-73343, a PLC inhibitor and its inactive analog (Cruzblanca et al. 1998). NG108-15 cells were pre-incubated with U-73122 for 30 min before electrophysiological experiments. Figure 1f shows that U-73122 (2 μM) blocks modulation of L current by BK, not by direct PKC activation with OAG, but a downstream step along the PLC

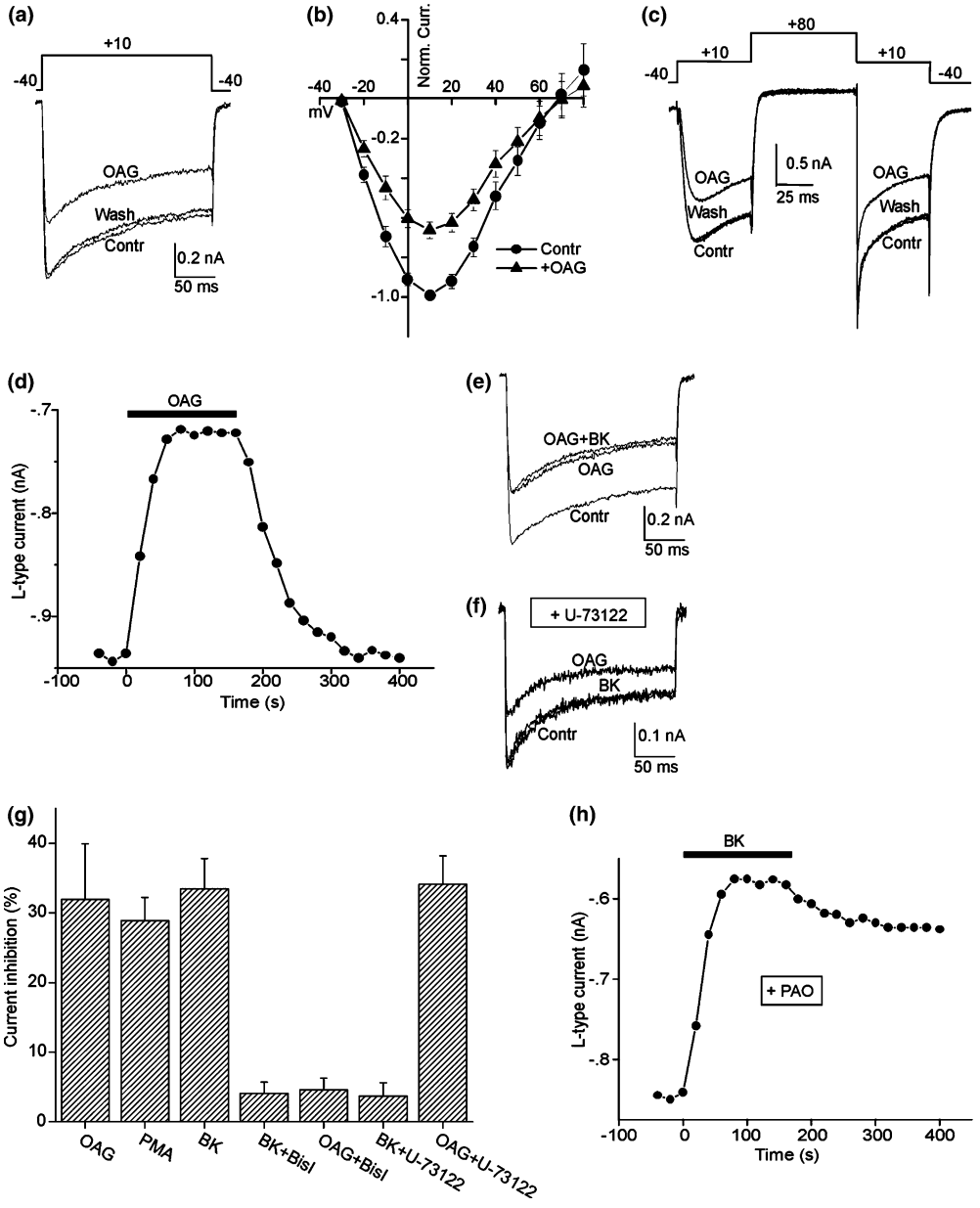

Fig. 1 BK-induced inhibition of L current is mimicked by PKC activators. **a** L currents recorded before (Contr), during (OAG) and after (Wash) application of the diacylglycerol analog OAG (5 μM) to a NG108-15 cell. **b** Mean L current amplitudes ($n=8$) plotted versus potential in control saline (*circles*) and in the presence of 5 mM OAG (*triangles*). **c** L current activity recorded at the test potential of +10 mV before and after a conditioning prepulse (+80 mV, 60 ms) during cell perfusion with control saline and during OAG application, showing no significant voltage-dependent recovery from OAG-induced inhibition. **d** Time-plot of OAG-mediated inhibition measured every 20 s before, during (OAG, *bar*) and after application of 5 μM OAG. The time constants of the on- and off-rate were 28.5 and 108.1 s respectively. **e** L current records obtained in control saline (Contr), during OAG application (OAG) and during application of OAG (5 μM) plus BK (100 nM), showing that the effects of OAG and BK are not additive. **f** L current records obtained in control saline (Contr), during BK application (100 nM) and during OAG application (5 μM) in a cell treated with the PLC inhibitor U-73122, showing that the inhibition of PLC prevents L current down modulation by BK but not by OAG. **g** The histogram shows the average percentages of L current inhibition measured at +10 mV during application of *1* OAG (5 μM), *2* the phorbol ester PMA (1 μM), *3* BK (100 nM), *4* BK plus the selective PKC inhibitor Bis I, *5* OAG plus Bis I, *6* BK plus the PLC inhibitor U-73122, and *7* OAG plus U-73122. **h** Time-plot of BK-mediated inhibition measured every 20 s before, during (BK, *bar*), and after application of 100 nM BK, following cell treatment with 20 μM PAO. The degree of BK-mediated inhibition was 31.7% and the current recovery during wash out was 20.8%

pathway. The average BK-mediated inhibition of L current was significantly lower in cells treated with U-73122 (3.7 ± 1.9%, $n=6$) than in control cells (Fig. 1g) or in cells treated with the inactive compound U-73343 (33.6 ± 4.9%, $n=3$). In contrast, the OAG modulation of L current was not blocked by the PLC inhibitor. OAG reduced the L current by 34.1 ± 4.1% ($n=6$) in cells treated with U-73122 (Fig. 1g). These results

demonstrate a clear role for PLC in coupling BK receptor activation to L current inhibition.

Because the PLC inhibitor blocked modulation of L current by BK, we tested whether BK would use IP_3 receptors in this intracellular signalling pathway. For this purpose we used the IP_3 receptor antagonist heparin and observed whether it blocked L current modulation by BK (Cruzblanca and Hille 1998). However, heparin (200 μg/ml) had no significant effect (the current inhibition was $31.5 \pm 4.5\%$, $n = 3$), making any involvement of IP_3 receptor activation in the modulation of L current by BK unlikely.

In a variety of cells, the receptor-mediated activation of PLC depletes PIP_2 in the plasma membrane, and the replenishment of PIP_2 requires lipid kinases, such as phosphoinositide 4-kinase (PI 4-K). Recent studies report that phenyl-arsine oxide (PAO) inhibits the replenishment of PIP_2 after receptor-mediated depletion by inhibiting PI 4-K (Sorensen et al. 1998; Varnai and Balla 1998; Suh and Hille 2002). Therefore, we asked if inhibition of PI 4-K by PAO might affect recovery of the L current from BK inhibition. As shown in Fig. 1h, following PAO application (20 μM in the bath), the recovery of current was only 20%. In another cell the action of PAO was similar, while in two more cells the recovery was almost completely blocked by PAO. Similar irreversible inhibition was also obtained in two cells treated with wortmannin (50 μM in the bath), another PI 4-K inhibitor. Despite their profound effect on recovery, neither PAO nor wortmannin affected the degree of L current inhibition during BK application.

Taken together, our results strengthen the hypothesis that BK receptor activation primes a PKC-dependent mechanism that mediates L current down modulation in NG108-15 cells, and the decrease in membrane PIP_2 concentration may play a crucial role in this intracellular signalling pathway.

In principle, the whole-cell L current can be described as the product of three factors:

$$I_L = N \times P_o \times i$$

where N is the total number of L-type channels, P_o is the probability of opening and i is the unitary current. In turn, P_o depends on both the fraction of the N channels that are active (available for opening during a depolarization), and on the probability that an active channel is open. Theoretically, the BK-mediated and PKC-dependent size reduction of the whole-cell L current could arise from alterations in any of the four factors (or any combination of these factors). Therefore, to discriminate between these possibilities, the effects of L-type Ca^{2+} channel inhibition by BK receptor stimulation and PKC activation were investigated further at the single channel level.

L-type channel activity in cell-attached patches

All experiments in cell-attached patches were carried out with the recording pipette containing 100 mM Ba^{2+}, 1 μM ω-conotoxin GVIA and 5 μM (±)-Bay K 8644. L-channel activity could only be well resolved under these experimental conditions, as shown in the sample tracings of Fig. 2a (see also the "Materials and methods" section). The amplitudes of unitary events were distributed around single gaussian functions with mean amplitudes, in the voltage range −20 to +40 mV, shown in Fig. 2b. The channel conductance (γ) was 18.8 ± 0.9 pS. The probability of opening (P_o), evaluated excluding the first and last closure of the channel and null sweeps, was voltage-dependent, with half-maximum at +10.6 mV and a maximum value of 0.39 above +40 mV (Fig. 2c). The mean open times (t_o) and mean closed times (t_c) at various voltages obtained from the arithmetic means of open and closed times of each patch (see "Materials and methods") were also voltage-dependent (Fig. 2d). Mean t_o increased weakly with voltage (an e-fold change for 41 mV) while mean t_c decreased more sharply with increasing voltage (an e-fold change for 20 mV). At 0 mV, mean t_o was 2.3 ms, which is a factor of ~1.4 smaller than that of L-channels in chromaffin cells (Carabelli et al. 2001) and, compared to N-channels, about six times larger than that found by Lee and Elmslie (1999) in sympathetic neurons, and about five times larger than that we measured in NG108-15 cells (not shown).

BK inhibits single L-type channel activity through a cytosolic pathway

We have shown that in NG108-15 cells, the whole-cell L current is inhibited by BK. The same is expected to occur at the single channel level. Furthermore, both the slow onset of BK-mediated inhibition and the mimetic inhibitory effect of OAG measured from whole-cell recordings (see above) support the idea of the involvement of a second messenger cascade rather than that of a membrane-delimited pathway in L-current inhibition, as proposed by Wilk-Blaszczak et al. (1996). We therefore tested whether BK could inhibit the activity of single L-channels through diffusible second messengers, by applying the agonist outside the patch. Figure 3 shows an example, which is representative of other three patches, where L channel activity was recorded at 0 mV during initial continuous superfusion outside the patch of control saline, followed by BK application (100 nM) and finally again by a control saline during wash-out. The B_2 receptors within the patch area were blocked by the selective antagonist HOE 140 (10 nM). P_o was monitored sweep by sweep, calculated by dividing the sum of the open times by the entire step duration, and plotted versus time. From Fig. 3 it is evident that P_o, relatively high during perfusion with control saline, decreased during BK application, and became high again during wash-out. Qualitatively similar results were obtained from three more patches. On average, the P_o values calculated by dividing the sum of the open times by the entire step duration and including null sweeps

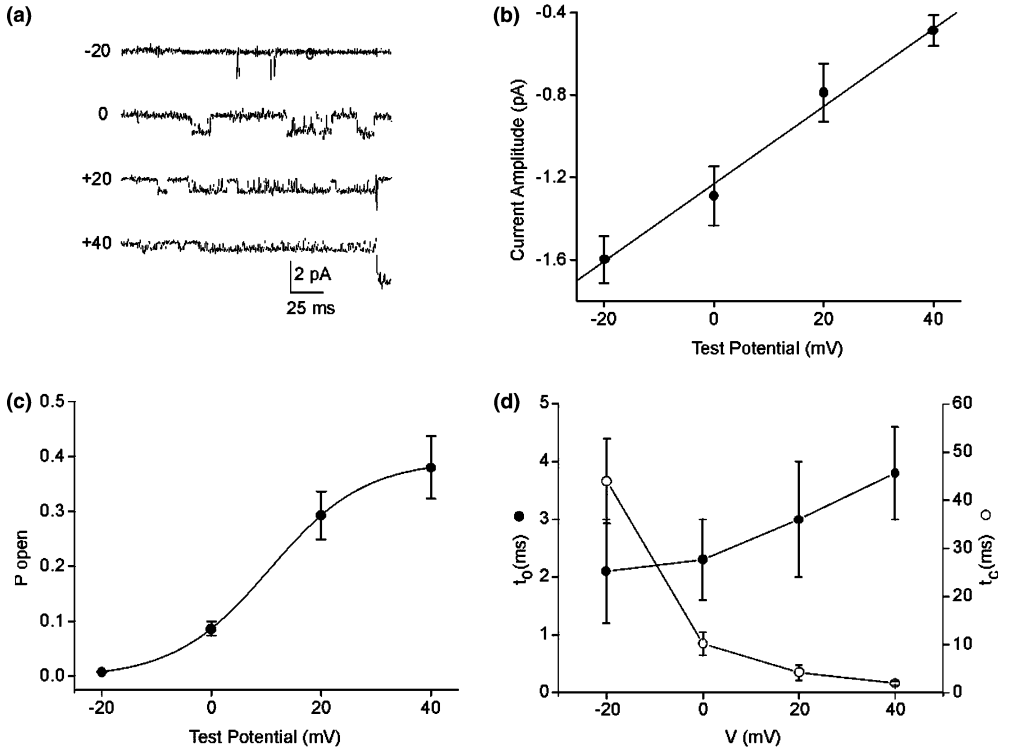

Fig. 2 Elementary properties of single L channels in NG108-15 cells. **a** Unitary L currents were recorded from −20 to +40 mV with 5 μM Bay K 8644 and 1 μM ω-conotoxin GVIA in the pipette solution; holding potential was −40 mV. **b** Mean unitary current amplitudes plotted against potential. The linear regression through the data points has a mean slope conductance of 18.8 pS ($n=5$). **c** Mean open probability plotted against potential obtained from five patches and evaluated excluding the first and last closure of the channel and null sweeps. Data points were fitted to a Boltzmann function $P_o = P_{max/(1+\exp(V1/2 \quad -V)/k)}$, where $P_{max}=0.39$, $V_{1/2}=10.6$ mV and $k=8.7$ mV. **d** Mean t_o (*filled circles*, *left scale*) and mean t_c (*open circles*, *right scale*) plotted between −20 and +40 mV as obtained from five patches. The two parameters were estimated by averaging the arithmetic means of the open and closed times of each patch

were 0.097 ± 0.012, 0.021 ± 0.009 and 0.088 ± 0.019 in control saline, during BK application, and during wash-out respectively ($n=4$), whereas the average P_o values of actively gating channels, evaluated excluding the first and last closure of the channel and null sweeps, were 0.111 ± 0.015, 0.072 ± 0.011 and 0.101 ± 0.022 in control saline, during BK application, and during wash-out respectively ($n=4$). This observation also demonstrates that BK inhibits single L-type current at single channel level, and can be taken as further evidence that diffusible second messengers are involved in the down-modulation of L-channels by BK in NG108-15 cells.

Bath application of BK has the great advantage that single channel recordings can be obtained under control conditions, in the presence of the agonist and during wash out, from the same cell, and it therefore demonstrates the specificity and reversibility of BK effects. However, the observation time (the number of sweeps and events to be collected) needed for quantitative analysis and for statistically-significant conclusions on BK effects at single channel level are hardly compatible with such a protocol. Therefore, the inhibitory effects of BK on single L-type channels were further and extensively studied in cell-attached patches distinct from controls, either by bath application of BK immediately after the cell-attached configuration was obtained, or by filling up the patch pipette with the agonist. The applicability of this procedure (the use of different cells for single channel recording with control saline and drug) was supported by the observation that in the whole-cell configuration the responsiveness to BK and the reversibility of the effect were high (sizeable and reversible inhibition was observed in more than 90% of the tested cells).

BK causes a significant increase in the null sweeps

Single channel sample tracings, obtained by membrane depolarization to 0 mV under control conditions and during BK application, are shown in Fig. 4. A simple inspection and comparison of the single-channel current traces obtained under control conditions (Fig. 4a) and in the presence of 100 nM BK (Fig. 4b), as well as of the average traces (Fig. 4a, b, bottom) indicates an overall reduction in L-type channel activity in the presence of BK. Interestingly, the inhibition of single channel gating by BK (~58%) was even larger than that measured during whole-cell recording (~30%). The only elucidation we have for this observation is that cell dialysis during the whole-cell configuration could deplete the inside of the cell of factors important for maximizing L-type current inhibition. Indeed, this explanation is not unlikely, considering the number of soluble cytoplasmic

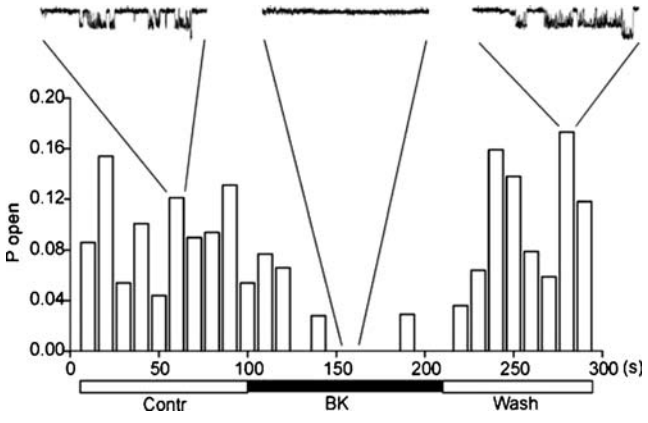

Fig. 3 Single L channel current inhibition by BK occurs through diffusible second messengers. To assess whether the L-channel inhibition requires any diffusible second messenger, BK was applied outside the patch, while the B_2 receptor antagonist HOE 140 (10 nM) was added to the patch pipette solution. The cell was initially perfused with control saline, then with a solution containing BK, and finally with control saline again for the time indicated by the *horizontal bars*. *Vertical bars* represent the P_o values corresponding to consecutive depolarizations at 0 mV. P_o was calculated sweep by sweep by dividing the sum of the open times by the entire step duration

molecular components possibly involved in this modulatory pathway.

This inhibitory effect of BK appears to arise from an increment of the time the channel spends in the closed state(s) rather than a decrement of the unitary current amplitude, the average value of which doesn't show significant changes in the presence of BK (control: -1.27 ± 0.21 pA, $n = 5$; + BK: -1.30 ± 0.18 pA, $n = 6$).

A detailed analysis of the parameters characterizing the kinetic properties of the L-type channel confirmed what we qualitatively observed by simple trace inspection by eye. In Fig. 5a–d, open- and closed-time distributions obtained from the fits of all pooled data at 0 mV in control saline and in the presence of BK are shown. Both open-time distributions were best fitted by double exponential functions (Fig. 5a, b), with mean open times that were not significantly different in the two experimental conditions: $t_{o1} = 0.9$ ms and $t_{o2} = 4.1$ ms in control conditions, while in the presence of BK $t_{o1} = 1.3$ ms and $t_{o2} = 5.3$ ms. The closed-time distributions were best fitted with three exponentials, as shown in Fig. 5c, d, with $t_{c1} = 0.3$ ms, $t_{c2} = 3.2$ ms and $t_{c3} = 166$ ms in control conditions and $t_{c1} = 0.3$ ms, $t_{c2} = 10.2$ ms and $t_{c3} = 131$ ms in the presence of BK respectively. Therefore, the major difference obtained from dwell times analysis was a factor 3.2 increase in the mean closed time t_{c2} during BK application. This could contribute to an overall decrease in the P_o. Indeed, the average P_o of actively gating channels, calculated from the pooled data at 0 mV excluding the first and last closure of the channel and null sweeps, was 0.155 in control saline and 0.110 during BK application, with a net reduction of 29%.

Another significant change in response to the agonist concerned the number of sweeps without activity. Null sweeps were found in every data set, by applying test pulses to 0 mV lasting 180 ms. However, in the presence of BK, the percentage of null sweeps increased significantly, by a factor 2.5 ($p < 0.04$), from $16.6 \pm 6.4\%$ in control saline ($n = 5$) to $41.5 \pm 11.1\%$ during BK application ($n = 4$), as shown in the histogram of Fig. 5e. Furthermore, null records appeared to be clustered in some of these data sets. We used run analysis to test whether the clustering of null sweeps was significant (Horn et al. 1984; Lee and Elmslie 1999). The results reached significance in three of four single L-channel patches in the presence of

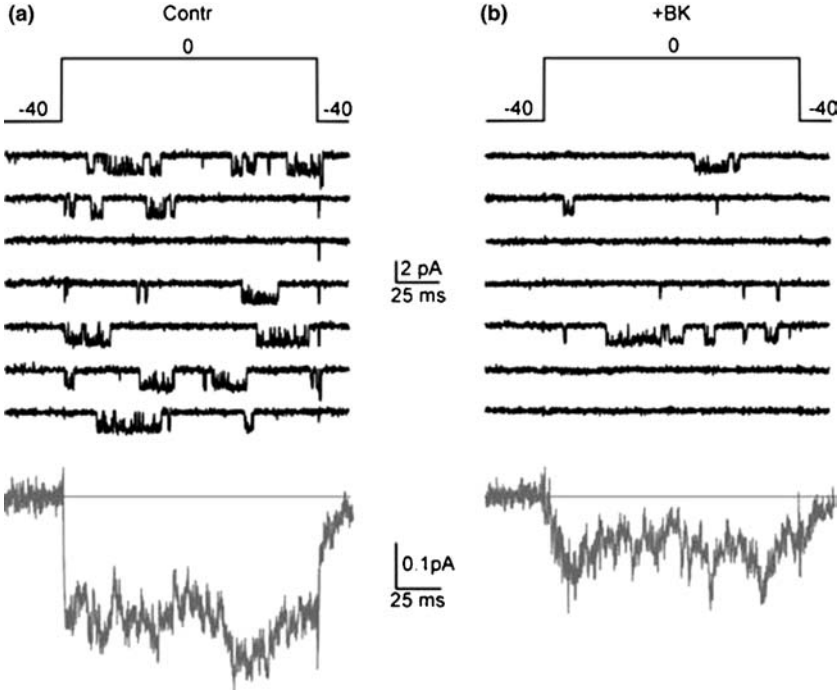

Fig. 4 Representative traces of L-channel activity recorded in different experimental conditions from cell-attached patches. **a** Single channel activity recorded in control conditions with pulses from −40 to 0 mV of 150 ms. The averaged current at the bottom (−0.27 pA) was calculated over 143 sweeps including null sweeps pooled from five patches. **b** BK (100 nM) was added to the control solution. The averaged current at the *bottom* had a lower amplitude (−0.11 pA) than the control ($n = 208$ sweeps including nulls, six patches). The patch pipette also contained 100 mM Ba^{2+}, the dihydropyridine agonist Bay K 8644 (5 μM) and the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (1 μM)

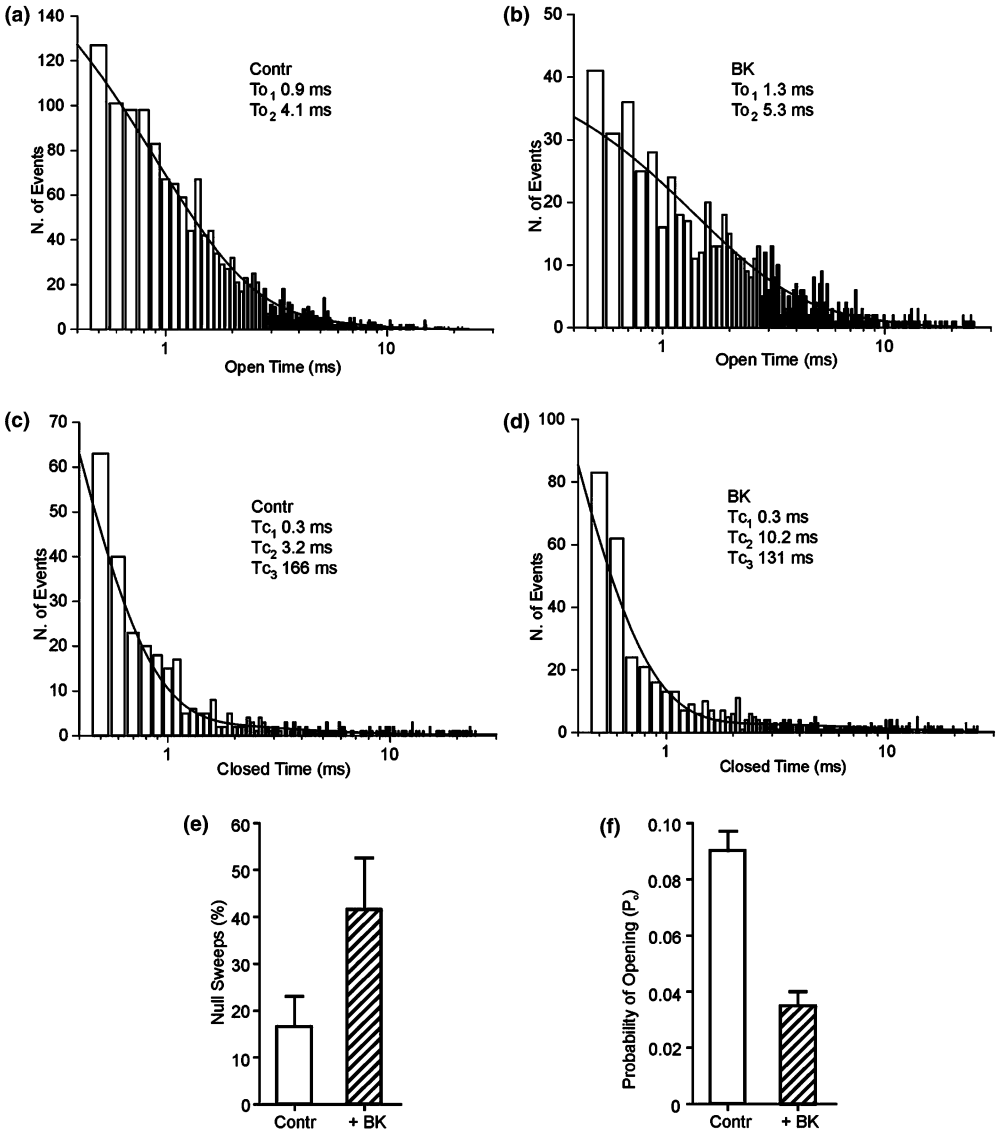

Fig. 5 BK causes an increase in null sweep frequency and an overall decrease in P_o Open and closed time distributions at 0 mV were obtained in control saline from 119 sweeps and five patches (**a–c**), and with BK from 105 sweeps and four patches (**b–d**). Open and closed distributions were fitted by two and three exponential functions respectively (*continuous curves*). **e** Mean percentage of null sweeps derived from five patches in control saline and from four patches with BK. *f* P_o values, calculated by dividing the sum of the open times by the entire step duration and including null sweeps, from five patches (control) and four patches (BK)

BK, with Z values of 1.6, 2.5 and 4.5 respectively. By contrast, run analysis indicated significant clustering ($L = 1.8$) in only one in five control patches, while null sweeps and active sweeps were randomly mixed in the remaining four patches. The increase in null sweeps with BK caused a consistent reduction in P_o. When calculated by dividing the sum of the open times by the entire step duration and including null sweeps, P_o dropped significantly, by 61% ($p < 0.03$) from 0.090 ± 0.007 in control saline ($n = 5$) to 0.035 ± 0.005 during BK application ($n = 4$), as shown in the histogram of Fig. 5f.

Single L-type channel down-modulation by OAG

Our whole-cell results strongly suggested the involvement of PKC in the BK-mediated L-current inhibition. Therefore we examined whether the PKC activator OAG could mimic the BK-mediated inhibition of L-channels in NG108-15 cells at the single channel level, by operating channel gating with a mechanism similar to that displayed following the application of BK. This was achieved by recording single channel activity from cell-attached patches with the patch pipette filled with the DAG analog. Figure 6a shows an example of tracings, representative of the other four patches, where single L channel activity was recorded at 0 mV in the presence of 5 μM OAG. Indeed, as with BK, a simple inspection of the average trace (Fig. 6a, bottom) and a comparison with that obtained under control conditions (Fig. 4a, bottom) indicated an overall reduction in L-type channel activity in the presence of OAG (56%), which, however, does not arise from a decrement of the unitary current amplitude, the average value of which does not shift significantly from the control (-1.24 ± 0.23 pA, $n = 4$). In the presence of OAG, another definite and significant difference to the control is an increase in the null sweeps, and as a consequence, a reduction in P_o, (Fig. 6b, c). In the presence of OAG the percentage of null sweeps increased by a factor of 2.4 ($p < 0.05$) to $39.4 \pm 10.8\%$

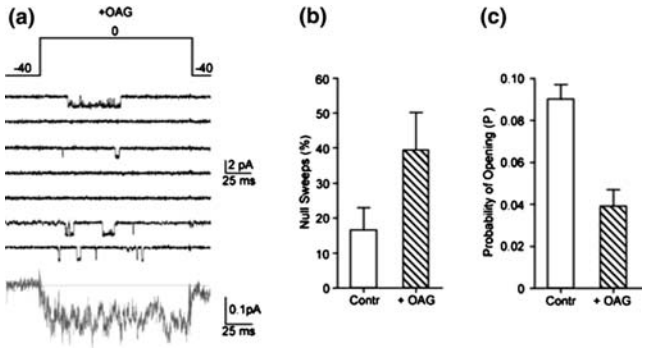

Fig. 6 L channel inhibition by BK is mimicked by OAG. **a** Representative traces of L-channel activity recorded at 0 mV from cell-attached patches in the presence of 5 μM OAG in the patch pipette. The averaged current at the bottom (−0.12 pA), calculated over 196 sweeps including null sweeps pooled from three patches, displays a lower amplitude than the control (−0.27 pA; see Fig. 4a bottom). **b** Mean percentage of null sweeps derived from five patches in control saline and from four patches with BK. **c** P_o values, calculated by dividing the sum of the open times by the entire step duration and including null sweeps, from five patches (control) and four patches (BK)

($n = 4$), while P_o (null sweeps included) reduced significantly by 57% ($p < 0.04$) to 0.039 ± 0.008 ($n = 4$).

Discussion

Evidence for a role of PKC in the regulation of L-type current in NG108-15 cells

By combining whole-cell and single channel recordings we have provided evidence supporting the idea that a non-membrane-delimited mechanism is responsible for the BK-mediated down-modulation of voltage-gated L-type Ca^{2+} channel currents, and that PKC is a likely intermediary for the BK-induced L current inhibition in NG108-15 cells. We have demonstrated that the BK effect on whole-cell L currents is mimicked by membrane-permeable activators of PKC, with a very similar degree of inhibition. Moreover, prior treatment of the cells with the DAG analog OAG prevents further reduction in L current by BK, suggesting a common intracellular pathway. The effective concentration ranges of the DAG analog OAG and of the phorbol ester PMA correspond well with concentrations used to activate PKC in cell-free assays (Ganong et al. 1986) and to modulate ion channel function in other cell types (Rane and Dunlap 1986; Boland et al. 1991). Additional evidence for the role of PKC is the ability of the selective PKC inhibitor Bis I (but not the inactive analog Bis V) to block the effects of the PKC activator and of BK itself on the L current.

The close correlation between the time courses for the effects of OAG on PKC-dependent phosphorylation (Kaibuchi et al. 1983) and of BK and OAG on L currents in NG108-15 cells also indicates that PKC may mediate the BK/OAG effect on L current. In platelets, OAG-stimulated phosphorylation was reported to be about half-maximal at 15 s and complete after 1 min (Kaibuchi et al. 1983). Similarly, we have found that for NG108-15 cells the onsets of BK- and OAG-mediated L current inhibition display time constants of about 30 s.

L-type channel modulation viewed through single-channel recordings

The previous results and the absence of voltage-dependent recovery from inhibition following application of a pre-pulse protocol suggest a mechanism for GPCR-dependent inhibition of L-type current that is completely different from the voltage-dependent down-modulation typical of N- or P/Q-type Ca^{2+} currents (Mathie et al. 1992; Bourinet et al. 1996; Bell et al. 2001) and produced by the direct binding of $G\beta\gamma$ to the channel (Herlitze et al. 1996; Ikeda 1996). Our further analysis at the level of unitary currents complements the prior study of whole-cell currents and furnishes suggestions about the molecular mechanism of L-type Ca^{2+} channel down-modulation in terms of a voltage-independent and PKC-dependent control of gating. Among the different parameters (unitary current, number of available channels and open probability) whose combination is responsible for the L current amplitude measured in whole-cell recording, the unitary current amplitude does not seem to be significantly reduced by BK or OAG application. It is also very unlikely that the expression and/or assembling of L-type channels at the plasma membrane may be affected in the relatively short application times of the two drugs, causing a significant reduction in the number of available channels. One major difference, found by the analysis of kinetic parameters, was a factor of 3.2 increase in one component of the closed time distribution (C2) following BK application. However, this could only contribute a maximum decrease in the P_o of 29%. We identified another property that, together with the increase of the closed time t_{c2}, substantially reduced P_o by about 60% in response to drug application, and this was an increase in the null sweeps. In particular, null sweeps were significantly clustered in the majority of our single L channel patches in the presence of BK. Clustering of null records has been reported for L-type calcium channels (Hess et al. 1984), skeletal muscle sodium channels (Horn et al. 1984), and N-type calcium channels (Lee and Elmslie 1999). These clustered null sweeps may be consistent with the hypothesis that they result from a mode of L channel gating from which the channel will not open (null P_o mode).

A classical model predicts that channel activities have an homogeneous gating pattern characterized by one set of kinetic parameter values (gating mode). Single channel recordings, however, have shown that Ca^{2+} channel activity may derive from a discrete number of gating modes, distinguishable for instance according to their probability of opening. Switching to different gating

modes with significantly different mean P_o values has been observed for both the L-type channel (Hess et al. 1984) and the N-type channel (Lee and Elmslie 1999). If we assume that modified channels exhibit long sojourns (hundreds of ms) in the null P_o mode, this would make the transitions from null to normal P_o mode rather unlikely within intervals of about 180 ms, which was the duration of our sweeps. This would originate BK- or OAG-modified ensemble currents of smaller size but with time courses similar to those of control currents, as we observed in the whole-cell configuration. In turn, at the single channel level, this would originate a sizeable increase in the number of null sweeps, as we observed in the cell-attached configuration.

Direct versus indirect action of PKC in L-type current down-modulation

We do not have definite indications of the final molecule(s) that alter L-type channel gating properties so that they exhibit increased null sweeps, and that are responsible for the BK-mediated channel down-modulation, but the contribution of PKC to L current inhibition may suggest that phosphorylation is required for L-type channel down-modulation. Indeed, phosphorylation has been postulated to regulate activation and inactivation properties of voltage-dependent Ca^{2+} channels and other channel types in other systems. In molluscan neurons, phosphorylation may play a role in Ca^{2+} channel inactivation (Chad and Eckert 1984). A dramatic reduction in channel P_o has been shown to be caused by direct activation of PKC in recombinant GIRK channels expressed in Xenopus oocytes (Stevens et al. 1999). Also, recent work suggests that phosphorylation of two threonine residues in the cardiac specific N-terminal domain is responsible for the inhibition of recombinant $Ca_v1.2$ channels by PKC in Xenopus oocytes (McHugh et al. 2000). Therefore, the involvement of PKC in L-current inhibition by BK may suggest that channel phosphorylation is implicated in generating the null gating mode, while dephosphorylation is needed to recover channel activity.

Our results could however suggest alternative scenarios. Although from our observations it seems reasonable to conclude that PKC must be considered an essential molecule in the signal cascade from BK receptor activation to L-type channel down-modulation, PKC might not directly interact with the channel. Alternatively, another key molecule that might be directly involved in the inhibition of L current is PIP_2. Increasing evidence suggests that PIP_2 is an important component of several intriguing intracellular signalling pathways. In particular, recent reports demonstrate that ion channel activity can be dramatically modulated by PIP_2 (Hilgemann et al. 2001). Our results might suggest a scenario similar to those proposed for the suppression of M current by muscarinic receptor activation (Suh and Hille 2002) or for GIRK channel inhibition by mechanical stretch (Zhang et al. 2004). BK receptors usually couple via the G proteins Gq/11 to PLC. Indeed, we have demonstrated the involvement of PLC, since the selective PLC inhibitor U-73122 blocks modulation of L current by BK but not by direct PKC activation with OAG. Therefore, it is reasonable to postulate that in response to BK, PLC may activate first, and in turn cleave PIP_2 into ip_3 and DAG. DAG then would activate PKC. We suggest that PKC plays the role of signal amplifier, making a positive-feedback circuit by enhancing the PIP_2 hydrolysis, the latter being responsible for L channel inhibition. Evidence for the involvement of PKC in hydrolysis of PIP_2 by PLC can be found in the work of Zhang et al. (2004). Namely, in GIRK channels, PIP_2 binding is thought to lead to the association of residues on the N and C termini with the inner surface of the plasma membrane, producing a channel conformation that favors activation. A reduction of PIP_2 content by PKC would be important for GIRK channel inhibition (Zhang et al. 2004). Likewise, a reduced interaction between PIP_2 and the L-type channel might contribute to modifying the gating pattern of unitary channels by favoring an increase in nulls and generating the null gating mode. This could represent an alternative (or complementary) mechanism to a direct PKC-dependent channel phosphorylation. Motifs similar to those involved in PIP_2 binding in GIRK channels have been found in N-type Ca^{2+} channels (Dolphin 2003). At the moment it is not known whether similar motifs are also present in neuronal L-type channels.

PIP_2 is also substrate for one class of PI 3-K whose immediate product might directly alter channel behavior. Such a mechanism, however, that does not imply PKC activation, is unlikely in our case for the following reasons: we have seen that (1) the degree of inhibition caused by BK is very similar to that obtained by direct PKC activation with OAG or phorbol esters; (2) OAG occluded the effect of BK, suggesting that a common, PKC-dependent converging mechanism mediates the BK-dependent down-modulation of the L current in NG108-15 cells; (3) wortmannin that, besides PI 4-K, inhibits also PI 3-K (Nakanishi et al. 1995), had no significant effect on BK-mediated L current inhibition. Rather than PI 3-K, PI 4-K, an enzyme involved in the formation of PIP_2 (Nakanishi et al. 1995), seems to play a role in L current modulation. Our observation that, following inhibition of PI 4-K, the recovery from BK inhibition was strongly reduced or abolished could suggest that regeneration of PIP_2 is required for recovery from Gq-coupled receptor-mediated inhibition of L-type calcium channels. Consistent with the idea that phosphorylation-dependent regeneration of PIP_2 is necessary for recovery from inhibition is the finding by Bannister et al. (2002) that AMP-PNP also prevents recovery from inhibition.

In summary, in the present study we have demonstrated that the neuropeptide BK is able to inhibit L-type channels in a non-membrane-delimited manner, involving PIP_2 hydrolysis and PKC activation. Fur-

thermore, we showed that inhibition by BK is mediated by a reduction in mean P_o caused principally by an increase in null sweeps. This may be due to a change in the gating properties of the channel complex caused by channel phosphorylation and/or channel modulation by PIP_2. A switch to null P_o gating could therefore be an additional process for controlling the Ca^{2+} entry during the action potential, which has consequences for Ca^{2+}-dependent processes such as neurotransmitter release, regulation of gene expression, dendritic development and synaptic plasticity. Specifically, BK is a neuropeptide involved in nociceptive pathways, and its receptors are localized in DRG neurons, the spinal cord, hypothalamus, thalamus and frontal cortex (Dray and Perkins 1993). BK has been reported to depolarize a subpopulation of DRG neurons in current-clamp recordings (Burgess et al. 1989). This depolarization (and spiking) is likely caused by the opening of a non-selective monovalent cation channel, and it in turn appears to be sufficient to induce the opening of L-type Ca^{2+} channels (Burgess et al. 1989). On the other hand, it has been also reported that BK blocks whole-cell Ca^{2+} currents in cultured rat DRG neurons (Ewald et al. 1989). What is the role of BK-mediated L-type channel inhibition in pain? If we assume that a fraction of the Ca^{2+} channels opened by the membrane depolarization are subsequently blocked by the exposure to BK, this inhibition of the L-current may serve a homeostatic role, damping the effects of depolarization on transmitter release. Furthermore, the findings of Burgess et al. (1989) raise the possibility that PKC regulates the monovalent cation channels that are opened upon exposure to BK. These data, along with the present study, suggest that the BK-induced depolarization measured in current clamp and the BK-induced reduction of Ca^{2+} current measured in voltage clamp are both mediated by PKC activation. If so, BK may provide a good example of how multiple cellular actions of a neurotransmitter can be produced by the activation of one kinase pathway.

Acknowledgements This work was supported by grants from Miur (FIRB 2001 and PRIN 2002) and INFM to M.T.

References

Akopian G, Walsh JP (2002) Corticostriatal paired-pulse potentiation produced by voltage-dependent activation of NMDA receptors and L-type Ca^{2+} channels. J Neurophysiol 87:157–165

Ashcroft FM, Proks P, Smith PA, Ammala C, Bokvist K, Rorsman P (1994) Stimulus-secretion coupling in pancreatic beta cells. J Cell Biochem 55:54–65

Bannister RA, Melliti K, Adams BA (2002) Reconstituted slow muscarinic inhibition of neuronal ($Ca_V1.2c$) L-type Ca^{2+} channels. Biophys J 83:3256–3267

Barg S, Ma X, Eliasson L, Galvanovskis J, Gopel SO, Obermuller S, Platzer J, Renstrom E, Trus M, Atlas D, Striessnig J, Rorsman P (2001) Fast exocytosis with few Ca^{2+} channels in insulin-secreting mouse pancreatic B cells. Biophys J 81:3308–3323

Bell DC, Butcher AJ, Berrow NS, Page KM, Brust PF, Nesterova A, Stauderman KA, Seabrook GR, Nurnberg B, Dolphin AC (2001) Biophysical properties, pharmacology, and modulation of human, neuronal L-type ($alpha_{1D}$, $Ca_V1.3$) voltage-dependent calcium currents. J Neurophysiol 85:816–827

Bley KR, Tsien RW (1990) Inhibition of Ca^{2+} and K^+ channels in sympathetic neurons by neuropeptides and other ganglionic transmitters. Neuron 2:379–391

Boland LM, Allen AC, Dingledine R (1991) Inhibition by bradykinin of voltage-activated barium current in a rat dorsal root ganglion cell line: role of protein kinase C. J Neurosci 11:1140–1149

Bourinet E, Soong TW, Stea A, Snutch TP (1996) Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. Proc Natl Acad Sci USA 93:1486–1491

Burgess GM, Mullaney I, McNeill M, Dunn PM, Rang HP (1989) Second messengers involved in the mechanism of action of bradykinin in sensory neurons in culture. J Neurosci 9:3314–3325

Carabelli V, Hernandez-Guijo JM, Baldelli P, Carbone E (2001) Direct autocrine inhibition and cAMP-dependent potentiation of single L-type Ca^{2+} channels in bovine chromaffin cells. J Physiol 532:73–90

Carbone E, Carabelli V, Cesetti T, Baldelli P, Hernandez-Guijo JM, Giusta L (2001) G-protein- and cAMP-dependent L-channel gating modulation: a manyfold system to control calcium entry in neurosecretory cells. Pflugers Arch 442:801–813

Chad JE, Eckert R (1984) Calcium domains associated with individual channels can account for anomalous voltage relations of Ca-dependent responses. Biophys J 45:993–999

Cho H, Jae Boum Youm, Earm YE, Won-Kyung H (2001) Inhibition of acetylcholine-activated K^+ current by chelerythrine and bisindolylmaleimide I in atrial myocytes from mice. Eur J Pharmacol 424:173–178

Colquhoun D, Hawkes AG (1995) Single channel recording. In: Sackmann B, Neher E (eds) The principles of the stocastic interpretation of ion channel mechanisms. Plenum, New York, pp 397–482

Connor M, Henderson G (1997) Bradykinin inhibition of N- and L-type calcium channel currents in NG108-15 cells. Neuropharmacol 36:115–124

Cruzblanca H, Duk-Su Koh, Hille B (1998) Bradykinin inhibits M current via phospholipase C and Ca^{2+} release from IP_3-sensitive Ca^{2+} stores in rat sympathetic neurons. Proc Natl Acad Sci USA 95:7151–7156

Davis PD, Elliott LH, Harris W, Hill CH, Hurst SA, Keech E, Kumar MK, Lawton G, Nixon JS, Wilkinson SE (1992) Inhibitors of protein kinase C. 2. Substituted bisindolylmaleimides with improved potency and selectivity. J Med Chem 35:994–1001

Dolmetsch RE, Pajvani U, Fife K, Spotts JM, Greenberg ME (2001) Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. Science 294:333–339

Dolphin AC (2003) G Protein modulation of voltage-gated calcium channels. Pharmacol Rev 55:607–627

Dray A, Perkins M (1993) Bradykinin and inflammatory pain. Trends Neurosci 16:99–104

Evans GJ, Pocock JM (1999) Modulation of neurotransmitter release by dihydropyridine-sensitive calcium channels involves tyrosine phosphorylation. Eur J Neurosci 11:279–292

Ewald DA, Pang IH, Sternweis PC, Miller RJ (1989) Differential G protein-mediated coupling of neurotransmitter receptors to Ca^{2+} channels in rat dorsal root ganglion neurons in vitro. Neuron 2:1185–1193

Finkbeiner S, Greenberg ME (1998) Ca^{2+} channel-regulated neuronal gene expression. J Neurobiol 37:171–189

Ganong BR, Loomis CR, Hannun YA, Bell RM (1986) Specificity and mechanism of protein kinase C activation by an 1,2-diacylglycerols. Proc Natl Acad Sci USA 83:1184–1188

Haymes AA, Kwan JW, Arena JP, Kass RS, Hinkle PM (1992) Activation of protein kinase C reduces calcium channel activity of GH3 pituitary cells. Am J Physiol 262:C1211–C1219

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca^{2+} channels by G-protein beta gamma subunits. Nature 380:258–262

Hernandez-Lopez S, Tkatch T, Perez-Garci E, Galarraga E, Bargas J, Hamm H, Surmeier DJ (2000) D2 dopamine receptors in striatal medium spiny neurons reduce L-type Ca^{2+} currents and excitability via a novel PLC[beta]1-IP_3-calcineurin- signaling cascade. J Neurosci 20:8987–8995

Hess P, Lansman JB, Tsien RW (1984) Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. Nature 311:538–544

Hidaka H, Inagaki M, Kawamoto S, Sasaki J (1984) Isoquinoline sulfonamides, novel and potent inhibitors of cyclic nucleotide protein kinase and protein kinase C. Biochemistry 23:5036–5041

Hilgemann DW, Feng SD, Nasuhoglu C (2001) The complex and intriguing lives of PIP2 with ion channels and transporters. Science STKE 111:RE19

Hockberger P, Toselli M, Swandulla D, Lux HD (1989) A dacylglycerol analogue reduces neuronal calcium currents independently of protein kinase C activation. Nature 338:340–342

Horn R, Vandenberg CA, Lange K (1984) Statistical analysis of single sodium channels: effects of N-bromoacetamide. Biophys J 45:323–335

Howe AR, Surmeier DJ (1995) Muscarinic receptors modulate N-, P-, and L-type Ca^{2+} currents in rat striatal neurons through parallel pathways. J Neurosci 15:458–469

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. Nature 380:255–258

Kaibuchi K, Takai Y, Sawamura M, Hoshijima M, Fujikura T, Nishizuka Y, (1983) Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. J Biol Chem 258:6701–674

Kamp TJ, Hell JW (2000) Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. Circ Res 87:1095–1102

Kasai H, Neher E (1992) Dihydropyridine-sensitive and omega-conotoxin-sensitive calcium channels in a mammalian neuroblastoma-glioma cell line. J Physiol 448:161–188

Kramer RH, Kaczmarek LK, Levitan ES (1991) Neuropeptide inhibition of voltage-gated calcium channels mediated by mobilization of intracellular calcium. Neuron 6:557–563

Lee HK, Elmslie KS (1999) Gating of single N-type calcium channels recorded from bullfrog sympathetic neurons. J Gen Physiol 113:111–124

Lee SB, Rhee SG (1995) Significance of PIP_2 hydrolysis and regulation of phospholipase C isozymes. Curr Opin Cell Biol 7:183–189

Mathie A, Bernheim L, Hille B (1992) Inhibition of N- and L-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. Neuron 8:907–914

McCullough LA, Egan TM, Westfall TC (1998) Neuropeptide Y inhibition of calcium channels in PC-12 pheochromocytoma cells. Am J Physiol 274:C1290–C1297

McDonough SI, Swartz KJ, Mintz IM, Boland LM, Bean BP (1996) Inhibition of calcium channels in rat central and peripheral neurons by omega-conotoxin MVIIC. J Neurosci 16:2612–2623

McHugh D, Sharp EM, Scheuer T, Catterall WA (2000) Inhibition of cardiac L-type calcium channels by protein kinase C phosphorylation of two sites in the N-terminal domain. Proc Natl Acad Sci USA 97:12334–12338

Nakanishi S, Catt KJ, Balla T (1995) Wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. Proc Natl Acad Sci USA 92:5317

Nishizuka Y (1984) Turnover of inositol phospholipids and signal transduction. Science 225:1365–1370

Osugi T, Imaizumi T, Mizushima A, Uchida S, Yoshida H (1987) Role of protein regulating guanine nucleotide binding in phosphoinositide breakdown and calcium mobilization by bradykinin in neuroblastoma × glioma hybrid NG108-15 cells: effects of pertussis toxin and cholera toxin on receptor-mediated signal transduction. Eur J Pharmacol 137:207–218

Oz M, Melia MT, Soldatov NM, Abernethy DR, Morad M (1998) Functional coupling of human L-type Ca^{2+} channels and angiotensin AT1A receptors coexpressed in xenopus laevis oocytes: involvement of the carboxyl-terminal Ca^{2+} sensors. Mol Pharmacol 54:1106–1112

Plummer MR, Logothetis DE, Hess P (1989) Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. Neuron 2:1453–1463

Rane, SG, Dunlap K (1986) Kinase C activator 1,2-oleylacetylglycerol attenuates voltage-dependent calcium current in sensory neurons. Proc Natl Acad Sci USA 83:184–188

Rane SG, Holz GGT, Dunlap K (1987) Dihydropyridine inhibition of neuronal calcium current and substance P release. Pflugers Arch 409:361–366

Sand O, Chen BM, Grinnell AD (2001) Contribution of L-type Ca^{2+} channels to evoked transmitter release in cultured Xenopus nerve-muscle synapses. J Physiol 536:21–33

Schwab Y, Mouton J, Chasserot-Golaz S, Marty I, Maulet Y, Jover E (2001) Calcium-dependent translocation of synaptotagmin to the plasma membrane in the dendrites of developing neurones. Brain Res Mol Brain Res 96:1–13

Sigworth FJ, Sine SM (1987) Data transformations for improved display and fitting of single-channel dwell time histograms. Biophys J 52:1047–1054

Stevens EB, Shah BS, Pinnock RD, Lee K (1999) Bombesin receptors inhibit G protein-coupled inwardly rectifying K+ channels expressed in Xenopus oocytes through a protein kinase C-dependent pathway. Mol Pharmacol 55:1020–1027

Striessnig J (1999) Pharmacology, structure and function of cardiac L-type Ca^{2+} channels. Cell Physiol Biochem 9:242–269

Suh BC, Hille B (2002) Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. Neuron 35:507–520

Tanabe T, Takeshima H, Mikami A, Flockerzi V, Takahashi H, Kangawa K, Kojima M, Matsuo H, Hirose T, Numa S (1987) Primary structure of the receptor for calcium channel blockers from skeletal muscle. Nature 328:313–318

Toselli M, Tosetti P, Taglietti V (1997) μ and δ opioid receptor activation inhibits ω-conotoxin-sensitive calcium channels in in a voltage- and time-dependent mode in the human neuroblastoma cell line SH-SY5Y. Pflugers Arch 433:587–596

tosetti P, Parente V, Taglietti V, Dunlap K, Toselli M (2003) Chick RGS2L demonstrates concentration-dependent selectivity for PTX-sensitive and -insensitive pathways that inhibit L-type Ca^{2+} channels. J Physiol 549:157–169

Toullec D, Pianettis P, Coste H, Belleverguel P, Grand-Perret T, Ajakanee M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamell L, Charon D, Kirilovsky J (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15778

Tsunoo A, Yoshii M, Narahashi T (1986) Block of calcium channels by enkephalin and somatostatin in neuroblastoma-glioma hybrid NG108-15 cells. Proc Natl Acad Sci USA 83:9832–9836

Varnai P, Balla T (1998) Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to Myo-[^{3}H]inositol-labeled phosphoinositide pools. J Cell Biol 143:501–510

Weisskopf MG, Bauer EP, LeDoux JE (1999) L-type voltage-gated calcium channels mediate NMDA-independent associative long-term potentiation at thalamic input synapses to the amygdala. J Neurosci 19:10512–10519

Wilk-Blaszczak MA, Singer WD, Belardetti F (1996) Three distinct G protein pathways mediate inhibition of neuronal calcium current by bradykinin. J Neurophysiol 76:3559–3562

Zhang L, Lee JK, John SA, Uozumi N, Kodama I (2004) Mechanosensitivity of GIRK channels is mediated by protein kinase C-dependent channel-phosphatidylinositol 4,5-bisphosphate interaction. J Biol Chem 279:7037–7047