

Presynaptic Inhibition via a Phospholipase C- and Phosphatidylinositol Bisphosphate-Dependent Regulation of Neuronal Ca^{2+} Channels

Stefan G. Lechner, Simon Hussl, Klaus W. Schicker, Helmut Drobny, and Stefan Boehm

Department of Pharmacology, Center of Biomolecular Medicine and Pharmacology, Medical University of Vienna, Vienna, Austria

Received May 19, 2005; accepted August 10, 2005

ABSTRACT

Presynaptic inhibition of transmitter release is commonly mediated by a direct interaction between G protein $\beta\gamma$ subunits and voltage-activated Ca^{2+} channels. To search for an alternative pathway, the mechanisms by which presynaptic bradykinin receptors mediate an inhibition of noradrenaline release from rat superior cervical ganglion neurons were investigated. The peptide reduced noradrenaline release triggered by K^{+} -depolarization but not that evoked by ATP, with Ca^{2+} channels being blocked by Cd^{2+} . Bradykinin also reduced Ca^{2+} current amplitudes measured at neuronal somata, and this effect was pertussis toxin-insensitive, voltage-independent, and developed slowly within 1 min. The inhibition of Ca^{2+} currents was abolished by a phospholipase C inhibitor, but it was not altered by a phospholipase A_2 inhibitor, by the depletion of intracellular Ca^{2+} stores, or by the inactivation of protein kinase C or Rho proteins. In whole-cell recordings, the reduction of Ca^{2+} cur-

rents was irreversible but became reversible when 4 mM ATP or 0.2 mM dioctanoyl phosphatidylinositol-4,5-bisphosphate was included in the pipette solution. In contrast, the effect of bradykinin was entirely reversible in perforated-patch recordings but became irreversible when the resynthesis of phosphatidylinositol-4,5-bisphosphate was blocked. Thus, the inhibition of Ca^{2+} currents by bradykinin involved a consumption of phosphatidylinositol-4,5-bisphosphate by phospholipase C but no downstream effectors of this enzyme. The reduction of noradrenaline release by bradykinin was also abolished by the inhibition of phospholipase C or of the resynthesis of phosphatidylinositol-4,5-bisphosphate. These results show that the presynaptic inhibition was mediated by a closure of voltage-gated Ca^{2+} channels through depletion of membrane phosphatidylinositol bisphosphates via phospholipase C.

Via changes in the strength of synaptic transmission, the nervous system can adapt to alterations in the environment, a phenomenon that is generally referred to as neuromodulation. In this respect, the modulation of transmitter release via presynaptic receptors is of utmost importance, and a plethora of neuromodulators act via presynaptic G protein-coupled receptors (GPCR). In most, if not all, types of synapses, the activation of GPCRs was found to lead to a pre-

synaptic inhibition of transmitter release, because activated G protein $\beta\gamma$ subunits directly interacted with voltage-activated Ca^{2+} channels (VACCs) and thereby reduced the Ca^{2+} influx required for vesicle exocytosis (Stevens, 2004). The precise mechanisms underlying the modulation of VACCs via GPCRs have been investigated in greatest detail in sympathetic neurons (Hille, 1994); there, the receptor-dependent activation of G proteins leads to an inhibition of Ca^{2+} currents (I_{Ca}) either via a direct, membrane-delimited and voltage-dependent interaction of G protein $\beta\gamma$ subunits with VACCs or via a second messenger system (Hille, 1994; Ikeda and Dunlap, 1999; Elmslie, 2003). In the experiments presented below, we used sympathetic neurons to delineate an

This study was supported by the "Virologiefonds" of the Medical University of Vienna and by the Austrian Science Fund (Fonds zur Förderung der Wissenschaftlichen Forschung P15797 and P17611).

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.105.014886.

ABBREVIATIONS: GPCR, G protein-coupled receptor; I_{Ca} , Ca^{2+} current; DEDA, 7,7-dimethyl-5,8-eicosadienoic acid; diC8-PIP₂, dioctanoyl phosphatidyl-4,5-bisphosphate; GF 109203X, bisindolylmaleimide I; IP₃, inositol trisphosphate; K_{M} channel, M-type K^{+} channel; LY 294,002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PIP₂, phosphatidylinositol 4,5-bisphosphate; PI3-kinase, phosphatidylinositol 3-kinase; PI4-kinase, phosphatidylinositol 4-kinase; PKC, protein kinase C; DTT, dithiothreitol; PAO, phenylarsine oxide; DMSO, dimethyl sulfoxide; PLA₂, phospholipase A_2 ; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; PTX, pertussis toxin; SCG, superior cervical ganglion; TTX, tetrodotoxin; U73122, 1-[6-[[[(17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione]; U73343, 1-[6-[[[(17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione; VACC, voltage-activated Ca^{2+} channel; UK 14304, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid acetoxymethyl ester.

example of presynaptic inhibition that relies on the modulation of VACCs through a second messenger system, but independently of a membrane-delimited action of G protein $\beta\gamma$ subunits.

In rat superior cervical ganglion (SCG) neurons, a large number of GPCRs including the prototypic α_2 -adrenoceptors mediate the voltage-dependent, membrane-delimited, $\beta\gamma$ -mediated inhibition of I_{Ca} , but only M_1 muscarinic and AT_1 angiotensin receptors were reported to reduce I_{Ca} in a voltage-independent manner via diffusible second messengers (Hille, 1994; Ikeda and Dunlap, 1999; Elmslie et al., 2003). These two latter receptors also use second messengers to inhibit M-type K^+ (K_M) channels (Hille, 1994). The underlying signal cascade remained obscure for decades but was recently evidenced to involve a phospholipase C (PLC)-dependent regulation of the membrane levels of phosphatidylinositol 4,5-bisphosphates (PIP_2 ; Suh and Hille, 2002; Zhang et al., 2003; Winks et al., 2005). While the present work was in progress, the same signaling pathway was reported to mediate the inhibition of VACCs in SCG neurons via M_1 receptors (Gamper et al., 2004). An inhibition of K_M channels in SCG neurons has also been observed when B_2 bradykinin receptors were activated (Jones et al., 1995), and this effect involved both the reduction of membrane PIP_2 and inositol trisphosphate-dependent increases in intracellular Ca^{2+} (Cruzblanca et al., 1998; Bofill-Cardona et al., 2000; Winks et al., 2005). Most recently, we found that bradykinin also caused a reduction of transmitter release from SCG neurons via presynaptic B_2 receptors and an inhibition of I_{Ca} (Edelbauer et al., 2005). However, the signaling cascade mediating the modulation of VACCs was not elucidated, and it remained obscure whether the inhibition of VACCs also occurred at presynaptic sites and thus was the basis for the reduction of transmitter release. Here, we first demonstrate that the inhibition of presynaptic VACCs is involved in the repression of transmitter release from rat SCG neurons by bradykinin and then provide evidence that the inhibition of both VACCs and transmitter release involves a PLC-dependent reduction in membrane PIP_2 .

Materials and Methods

Primary Cultures of Rat Superior Cervical Ganglion Neurons. Primary cultures of dissociated SCG neurons from neonatal rats were prepared as described previously (Boehm, 1999). Ganglia were dissected from 2- to 6-day-old Sprague-Dawley rat pups that had been killed by decapitation in accordance with the rules of the university animal welfare committee. After incubation in collagenase (1.5 mg/ml; Sigma-Aldrich, Vienna, Austria) and dispase (3.0 mg/ml; Roche Diagnostics, Mannheim, Germany) for 45 min at 36°C, ganglia were trypsinized (0.25% trypsin; Worthington Biochemicals, Lakewood, NJ) for 20 min at 36°C, dissociated by trituration, and resuspended in Dulbecco's modified Eagle's medium (Invitrogen, Vienna, Austria) containing 2.2 g/l glucose, 10 mg/l insulin, 25,000 IU/l penicillin, 25 mg/l streptomycin (Invitrogen), 50 μ g/l nerve growth factor (R&D Systems, Minneapolis, MN), and 5% fetal calf serum (Invitrogen). Cells were plated either onto 5-mm discs (approximately 40,000 cells per disc) for [3 H]noradrenaline release or onto 35-mm culture dishes for electrophysiology. All tissue culture plastic was coated with rat tail collagen (Biomedical Technologies, Stoughton, MA). Cells were kept in a humidified 5% CO_2 atmosphere at 36°C for up to 7 days, and one half of the medium was exchanged twice during this culture period. At 1 to 2 days before experiments, fresh medium without serum was added.

Determination of [3 H]Noradrenaline Release. The release of [3 H]noradrenaline was determined as described before (Boehm, 1999). Cultures were labeled with 0.05 μ M [3 H]noradrenaline (specific activity, 71.7 Ci/mmol) in culture medium supplemented with 1 mM ascorbic acid at 36°C for 1 h. After labeling, culture discs were transferred to small chambers and superfused with a buffer containing 120 mM NaCl, 6.0 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 20 mM glucose, 10 mM HEPES, 0.5 mM fumaric acid, 5.0 mM sodium pyruvate, 0.57 mM ascorbic acid, and 0.001 mM desipramine, adjusted to pH 7.4 with NaOH. Superfusion was performed at 25°C at a rate of approximately 1.0 ml/min. The collection of 4-min superfusate fractions was started after a 60-min washout period. Tritium overflow was evoked during two consecutive stimulation periods (S1 and S2) by the inclusion of either 0.3 mM ATP or 40 mM KCl (NaCl was reduced accordingly to maintain isotonicity) in the buffer for 60 s. Radioactivity released in response to electrical field stimulation from rat sympathetic neurons after labeling with tritiated noradrenaline and under conditions similar to those of the present study had been shown previously to consist predominantly of the authentic transmitter and to contain only small amounts ($\leq 15\%$) of metabolites (Schwartz and Malik, 1993). Hence, the outflow of tritium measured in this study was assumed to reflect primarily the release of noradrenaline and not that of metabolites.

Tetrodotoxin (TTX; 0.1 μ M), $CdCl_2$ (100 μ M), and thapsigargin (0.3 μ M), if appropriate, were added to the superfusion buffer after 50 min of superfusion (i.e., 10 min before the start of sample collection). Bradykinin (1 μ M) and UK 14304 (1 μ M) were added to the superfusion buffer 2 min before and phenylarsine oxide (10 μ M) and dithiothreitol (1 mM) 4 min before the second stimulation period. At the end of experiments, the radioactivity remaining in the cells was extracted by immersion of the discs in 1.2 ml of 2% (v/v) perchloric acid followed by sonication. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting (Packard Tri-Carb 2100 TR; PerkinElmer Life and Analytical Sciences, Boston, MA).

Electrophysiology. I_{Ca} of sympathetic neurons was determined as described previously (Boehm et al., 1996). Currents were recorded at room temperature (20–24°C) from single SCG neurons in vitro using an Axopatch 200B amplifier and the Pclamp 6.0 hard- and software (Axon Instruments Inc., Union City, CA). Currents were low-pass filtered at 5 kHz, digitized at 10 to 50 kHz, and stored on an IBM-compatible computer. Traces were analyzed offline by the Clampfit 8.1 program (Axon Instruments). Patch electrodes were pulled (Flaming-Brown puller; Sutter Instruments, Novato, CA) from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany). For perforated-patch recordings, electrodes were front-filled with a solution consisting of 130 mM CsCl, 20 mM tetraethylammonium chloride, 0.24 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, and 5 mM EGTA, adjusted to pH 7.3 with KOH, and were then back-filled with the same solution containing 200 μ g/ml amphotericin B (in 0.8% DMSO), which yielded tip resistances of 2 to 3 M Ω . For whole-cell recordings, electrodes were filled with the solution used for front-filling, which additionally contained 2 mM magnesium ATP and 2 mM sodium GTP. Unless stated otherwise, all experiments were performed in the perforated-patch configuration. The external solution contained 120 mM NaCl, 20 mM tetraethylammonium chloride, 3 mM KCl, 2 mM $MgCl_2$, 5 mM $CaCl_2$, 20 mM glucose, and 10 mM HEPES, adjusted to pH 7.3 with KOH. This combination of solutions results in small liquid junction potentials of approximately +2 mV, which, however, were neglected. In a few experiments, 5 mM Ba^{2+} was used instead of Ca^{2+} as the charge carrier. Drugs were applied via a DAD-12 drug application device (Adams and List, Westbury, NY), which permits a complete exchange of solutions surrounding the cells under investigation within less than 100 ms (Boehm, 1999).

Unless stated otherwise, I_{Ca} was elicited every 15 s by 30-ms depolarizations from a holding potential of -80 to $+10$ mV. Leakage currents were corrected for by an online leak subtraction protocol,

which applies four hyperpolarizing pulses before the depolarization to +10 mV to determine the extent of leakage. The extent of I_{Ca} inhibition by bradykinin was quantified according to the equation % inhibition = $100 - 100 \times (B_1 + B_2)/(C_1 + C_2)$, where B_1 and B_2 are the peak current amplitudes determined 45 and 60 s after the start of bradykinin application, and C_1 and C_2 are the amplitudes of control currents measured directly before the bradykinin application. Recovery from inhibition was calculated using the equation: % recovery = $100 \times [(W_1 + W_2) - (B_1 + B_2)]/[(C_1 + C_2) - (B_1 + B_2)]$, where W_1 and W_2 are the current amplitudes measured 135 and 150 s after the start of bradykinin washout ($B_{1,2}$, $C_{1,2}$, and $W_{1,2}$ are indicated in Fig. 2A).

To determine the voltage dependence of inhibition, currents were elicited by a double-pulse voltage protocol (illustrated in Fig. 2D): cells were clamped at -80 mV; every 15 s, a 35-ms depolarization to +10 mV ($I_{Ca,-pp}$) was applied, followed by a 3-s waiting period at -80 mV, a 20-ms prepulse to +80 mV, a 10-ms repolarization to -80 mV, and finally another 35-ms depolarization to +10 mV ($I_{Ca,+pp}$). Facilitation was calculated as the ratio of I_{Ca} amplitudes measured before and after the prepulse, respectively, by using the equation: prepulse facilitation = $I_{Ca,+pp}/I_{Ca,-pp}$.

Statistics. All data represent arithmetic means \pm S.E.M.; n represents the number of culture discs in [³H]noradrenaline-release experiments and the number of single neurons in electrophysiological experiments. Statistical significances between data points were evaluated by the nonparametric Mann-Whitney test.

Materials. (–)-[Ring-2,5,6-³H]noradrenaline was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA); bradykinin, TTX, pertussis toxin (PTX), U73122, U73343, thapsigargin, *Clostridium difficile* toxin B, 7,7-dimethyl-5,8-eicosadienoic acid (DEDA), LY

294,002, wortmannin, and UK 14304 were from Sigma-Aldrich; phorbol-12-myristate-13-acetate (PMA) and GF 109203X were from Calbiochem (Bad Soden, Germany); BAPTA-AM was from Molecular Probes (Eugene, OR); and dioctanoyl phosphatidyl-4,5-bisphosphate (diC8-PIP₂) was from Cayman Chemicals (Ann Arbor, MI).

Results

Bradykinin Reduces the Release of [³H]Noradrenaline Evoked by K⁺ but Not That Evoked by ATP in the Presence of Cd²⁺. SCG neurons labeled with [³H]noradrenaline steadily released small amounts of tritium into the superfusion buffer when excess radioactivity had been removed during a 60-min washout period. The buffer contained 0.1 μ M TTX to isolate drug effects that occurred at nerve terminals from those at remote sites, such as axons or neuronal somata, and to thereby prevent the release-stimulating action of bradykinin (Boehm and Huck, 1997; Edelbauer et al., 2005). The spontaneous release of radioactivity into the superfusion buffer per 4-min collection period amounted to $1.03 \pm 0.19\%$ of total tritium in the cultures ($n = 30$), which corresponded to 0.22 ± 0.04 nCi.

Depolarization of SCG neurons by high K⁺ concentrations induces the release of previously incorporated [³H]noradrenaline by eliciting transmembrane Ca²⁺ influx via VACCs, which can be abolished by 100 μ M Cd²⁺. Application of 0.3 mM ATP in the continuing presence of 100 μ M Cd²⁺, in contrast, causes Ca²⁺ entry via P2X receptors and thereby

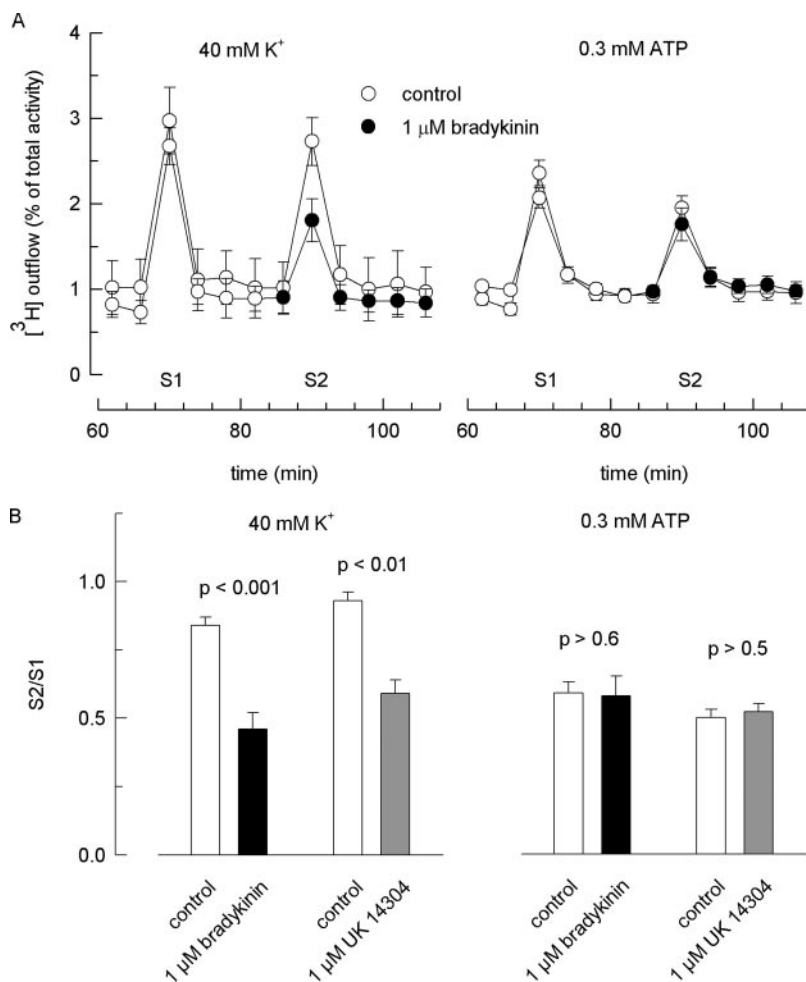


Fig. 1. Inhibition of K⁺-evoked but not ATP-evoked noradrenaline release from SCG neurons by bradykinin: comparison with an α_2 adrenergic agonist. After loading with [³H]noradrenaline, neurons were superfused, and subsequent to a 1-h washout period, 4-min fractions of superfusate were collected. After 68 (S1) and 88 (S2) min of superfusion, the cultures were stimulated for 60 s by the presence of either 40 mM K⁺ (Na⁺ was reduced accordingly) or 0.3 mM ATP. When ATP was used as stimulus, the buffer contained 100 μ M Cd²⁺ to block VACCs. A, time course of tritium outflow as a percentage of the total radioactivity in the cultures. Bradykinin (1 μ M) was present from minute 86 onward in the cultures represented by filled symbols; $n = 3$. B, a comparison of the effects of bradykinin (1 μ M) and the α_2 agonist UK 14304 (1 μ M) on K⁺- and ATP-evoked tritium overflow. The receptor agonists were present as shown for bradykinin in A, and the resulting S2/S1 ratios are shown; $n = 6$ to 12.

triggers [^3H]noradrenaline release independently of VACCs (Boehm, 1999). Exposure to either 40 mM KCl (NaCl was reduced to maintain isotonicity) or 0.3 mM ATP in the continuing presence of Cd^{2+} during two consecutive periods of stimulation (S1 and S2) caused reproducible increases in tritium release (Fig. 1A), which lacked in the absence of extracellular Ca^{2+} (data not shown; Boehm, 1999). When bradykinin (1 μM) was present during the second period of K^+ stimulation (S2), the amount of released tritium was reduced (Fig. 1A). Therefore, the ratio of the amount of tritium efflux triggered during the two stimulation periods (S2/S1) was decreased from 0.84 ± 0.03 ($n = 9$) in the absence of the peptide to 0.46 ± 0.06 ($n = 9$; $p < 0.01$) in its presence. In contrast, when ATP was used to stimulate tritium efflux, bradykinin failed to cause an inhibition, and the S2/S1 ratio remained unchanged [0.59 ± 0.04 in the absence ($n = 12$) and 0.58 ± 0.07 in the presence of bradykinin ($n = 9$); Fig. 1B].

α_2 -Adrenoceptors are well known to reduce noradrenaline release from sympathetic neurons through an inhibition of VACCs (Boehm and Kubista, 2002), and the α_2 -adrenergic agonist UK 14304 causes presynaptic inhibition in SCG neurons only when VACCs are involved in excitation-secretion coupling (Boehm and Huck, 1995). In accordance with that observation, 1 μM UK 14304 diminished the S2/S1 ratio of K^+ -evoked but not that of ATP-evoked tritium efflux (Fig.

1B). Hence, activation of both presynaptic bradykinin B_2 and α_2 -adrenergic receptors only reduced [^3H]noradrenaline release, when transmembrane Ca^{2+} entry occurred via VACCs. The presynaptic inhibition of transmitter release was presumably mediated by an inhibition of VACCs.

Bradykinin Induces a Slow, Voltage-Independent, and PTX-Resistant Inhibition of I_{Ca} . The above results demonstrate that the inhibition of VACCs is the mechanism by which bradykinin reduces sympathetic transmitter release. We therefore investigated the signal cascade by which bradykinin inhibits VACCs. To this end, we first determined some of the basic parameters of the inhibitory action of bradykinin on I_{Ca} and compared them with those of the α_2 -adrenergic agonist UK 14304. Evidence has been provided that GPCRs may use different signaling pathways to regulate I_{Ca} in rat SCG neurons, depending on the recording technique (Filippov et al., 2003). Therefore, the initial set of experiments was performed in the whole-cell and perforated-patch configuration. In whole-cell, 1 μM bradykinin reduced I_{Ca} by $45.04 \pm 5.16\%$ ($n = 11$), and the reduction of current amplitudes was maximal after 45 to 60 s (Fig. 2A). For comparison, UK 14304 (10 μM) reduced whole-cell I_{Ca} by $47.95 \pm 5.23\%$ ($n = 13$), and this effect was maximal after <15 s (data not shown). In the perforated-patch configuration, the reduction of I_{Ca} by 1 μM bradykinin was again

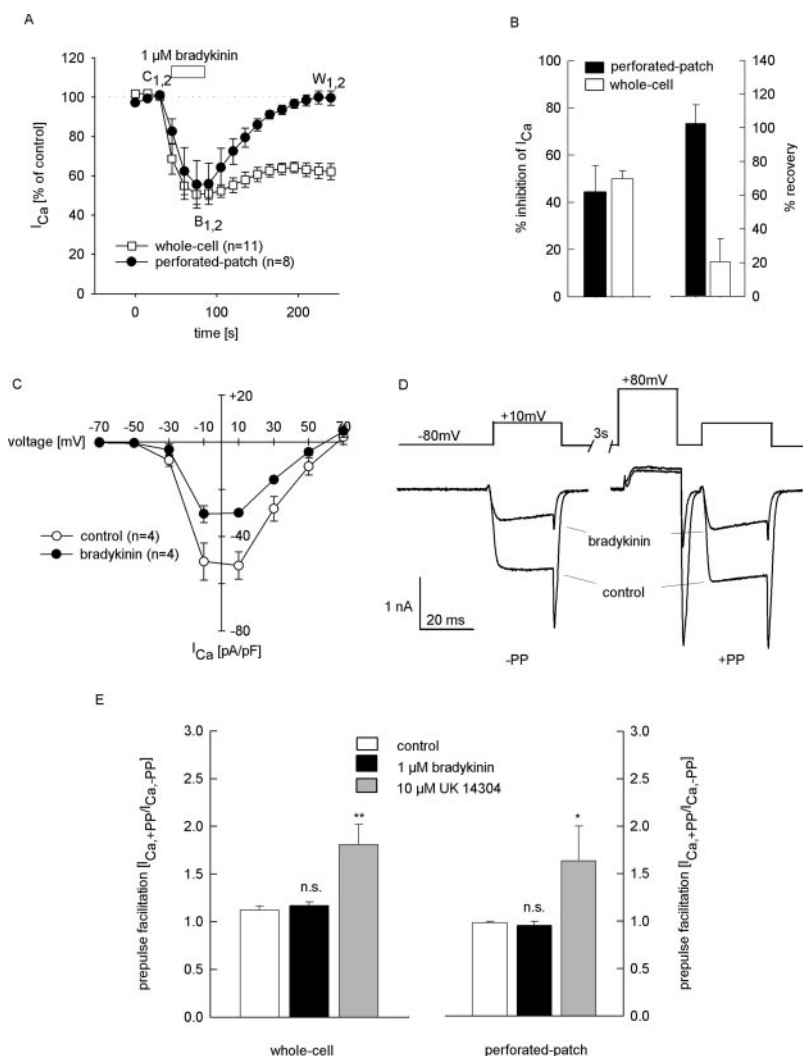


Fig. 2. Inhibition of I_{Ca} by bradykinin in whole-cell and perforated-patch recordings: time course and voltage-dependence. Ca^{2+} currents were elicited once every 15 s by depolarizations from -80 to $+10$ mV (unless indicated otherwise), and bradykinin was present for 1 min (as shown in A). Inhibition of peak current amplitudes was calculated as $\% \text{ inhibition} = 100 - 100 \times (B_1 + B_2) / (C_1 + C_2)$, and recovery from the inhibition was calculated as $\% \text{ recovery} = 100 \times [(W_1 + W_2) - (B_1 + B_2)] / [(C_1 + C_2) - (B_1 + B_2)]$. A, these points in time and the time course of current amplitudes are shown in either whole-cell ($n = 11$) or perforated-patch ($n = 8$) recordings. B, the quantification of the inhibition and recovery of current amplitudes for the same cells as in A. C, a current-voltage curve for whole-cell currents evoked by 30-ms depolarizations from -80 mV to the indicated voltages in the absence and presence of 1 μM bradykinin. D, the pulse protocol for the determination of current facilitation by depolarizing prepulses and the resulting perforated-patch current traces determined in the absence (control) and presence of 1 μM bradykinin. E, the prepulse facilitation in the absence and presence of either 1 μM bradykinin or 10 μM UK 14304 in either whole-cell ($n = 6$) or perforated-patch ($n = 5$) recordings. Significant differences versus the corresponding controls are shown via **, $p < 0.01$ and *, $p < 0.05$, respectively; n.s., no significant difference.

maximal after 45 to 60 s and amounted to $44.28 \pm 11.2\%$ ($n = 8$) inhibition (Fig. 2A). In such recordings, UK 14304 reduced I_{Ca} by $49.87 \pm 11.13\%$ ($n = 6$), and the effect was maximal after <15 s (data not shown). With respect to the recovery from the inhibition by bradykinin, however, there were significant differences between the two recording techniques: during a 150-s washout period, I_{Ca} only partially recovered from the inhibition in whole-cell recordings ($24.95 \pm 14.78\%$ recovery), whereas the inhibition was fully reversible in perforated-patch recordings ($102.39 \pm 11.85\%$ recovery) (Fig. 2, A and B). For comparison, the inhibition by UK 14304 was entirely reversible within 30 s in both types of recordings (data not shown).

The inhibition of I_{Ca} in SCG neurons via the membrane-delimited pathway is voltage-dependent and thus decreases the more the cell is depolarized (Hille, 1994). In the present experiments, however, the reduction of I_{Ca} amplitudes by bradykinin was approximately the same at voltages between -30 and $+50$ mV (Fig. 2C). The voltage-dependent membrane-delimited inhibition of I_{Ca} is also characterized by a slowing of activation kinetics, which is attenuated by brief depolarizing prepulses, a phenomenon called "prepulse facilitation" (Hille, 1994). The prepulse facilitation of the bradykinin inhibition was assessed by a double-pulse protocol and compared with that of the α_2 -adrenergic inhibition caused by UK 14304. The bradykinin inhibition was neither accompanied by a slowing of activation kinetics nor attenuated by the prepulse (Fig. 2D). Therefore, the values of prepulse facilitation in the presence of bradykinin were not different from those in the absence of the peptide. In contrast, in the presence of $10 \mu\text{M}$ UK 14304, a marked prepulse facilitation was observed. The extent of prepulse facilitation in the absence or presence of receptor agonists was not different between whole-cell and perforated-patch recordings (Fig. 2E). When neurons had been treated for 24 h with 100 ng/ml PTX, the inhibition by UK 14304 was completely abolished ($0.94 \pm 1.18\%$ inhibition; $n = 7$), but the inhibition by bradykinin remained unaffected ($44.52 \pm 6.45\%$ inhibition; $n = 7$).

PLC, but None of Its Downstream Effector Systems, Is Required for the Inhibition of I_{Ca} . In rat SCG neurons, the actions of bradykinin are commonly mediated by $G_{q/11}$ proteins and PLC- β (Haley et al., 2000; Scholze et al., 2002). The inhibition of PLC in SCG neurons by U73122 prevents the formation of inositol phosphates in response to bradykinin, an effect that is not observed with the inactive analog U73343 (Bofill-Cardona et al., 2000). Here, the inhibition of I_{Ca} , as determined in perforated-patch recordings, was largely attenuated when neurons had been incubated in $3 \mu\text{M}$ U73122 for 30 min. An equivalent incubation in U73343, however, did not alter the I_{Ca} inhibition by bradykinin (Fig. 3).

Via PLC- β , bradykinin triggers the synthesis of inositol trisphosphate (IP_3) and diacylglycerol, which in turn cause Ca^{2+} release from the endoplasmic reticulum and activation of protein kinase C (PKC) (Cruzblanca et al., 1998; Scholze et al., 2002), respectively. However, when neurons had been treated with the Ca^{2+} ATPase inhibitor thapsigargin ($0.3 \mu\text{M}$) for 30 min to deplete the intracellular Ca^{2+} stores (Foucart et al., 1995), the peptide reduced I_{Ca} to the same extent as under control conditions (Fig. 3). Likewise, in neurons incubated for at least 30 min in $3 \mu\text{M}$ concentration of the cell-permeant Ca^{2+} chelator BAPTA-AM (followed by a 30-

min incubation in regular buffer to permit hydrolysis of the acetoxymethylester), bradykinin diminished current amplitudes again by approximately 50% (Fig. 3). However, these two latter manipulations did abolish the inhibition of I_M by bradykinin (data not shown; Bofill-Cardona et al., 2000). These results indicate that the I_{Ca} inhibition did not require the release of Ca^{2+} from its stores into the cytosol. To investigate whether Ca^{2+} entry via VACCs was essential, whole-cell recordings were performed with Ba^{2+} instead of Ca^{2+} as charge carrier. In these experiments, bradykinin reduced current amplitudes by only $15.50 \pm 3.02\%$ ($n = 9$) compared with the $36.37 \pm 5.05\%$ inhibition with Ca^{2+} as charge carrier ($n = 7$; $p < 0.01$). Hence, the flow of Ca^{2+} ions through VACCs was required to permit maximal current inhibition by bradykinin.

Exposure of the neurons to the PKC inhibitor GF 109203X ($0.3 \mu\text{M}$ for 30 min), which abrogates the excitatory actions of bradykinin in SCG neurons (Scholze et al., 2002), failed to alter the reduction of I_{Ca} . Therefore, a pretreatment with $1 \mu\text{M}$ PMA for 24 h, which eliminates classic and novel PKC isoforms from SCG neurons (Scholze et al., 2002), did not attenuate the inhibitory action of bradykinin (Fig. 3).

In neuroblastoma-glioma hybrid (NG108-15) cells, bradykinin inhibits I_{Ca} via the monomeric G proteins Rac1 and/or Cdc42 (Wilk-Blaszczak et al., 1997). To test for a role of this signaling pathway, cultures were treated for at least 6 h with 50 ng/ml *C. difficile* toxin B, which inactivates members of the Rho protein family, such as Rac1 and Cdc42, by glycosylation (Just et al., 1995). However, the inhibitory action of bradykinin remained unaffected (Fig. 3), although the toxin

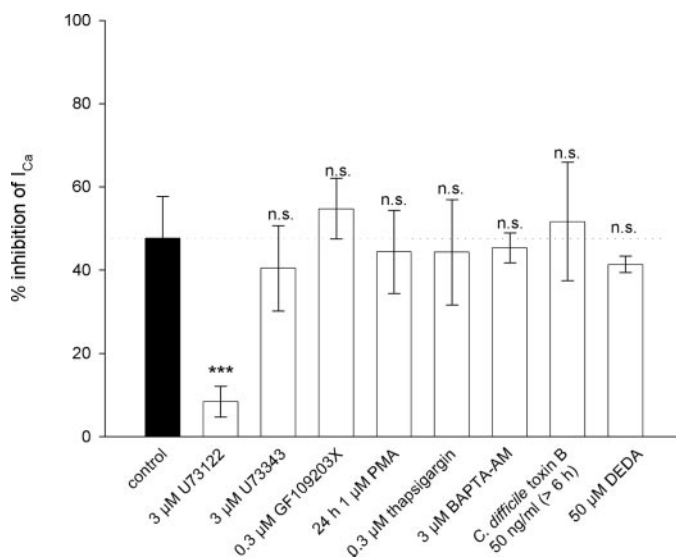


Fig. 3. Inhibition of I_{Ca} by bradykinin in perforated-patch recordings: roles of PLC, PKC, PLA₂, intracellular Ca^{2+} , and Rho proteins. Ca^{2+} currents were elicited by depolarizations from -80 to $+10$ mV once every 15 s. Bradykinin was present for 1-min periods, and the inhibition of current amplitudes was calculated as shown in Fig. 2A. Before the recording of currents, neurons had been incubated in $3 \mu\text{M}$ U73122, $3 \mu\text{M}$ U73343, $0.3 \mu\text{M}$ thapsigargin, $3 \mu\text{M}$ BAPTA-AM (followed by a 30-min incubation in regular buffer), or $0.3 \mu\text{M}$ GF 109023X, each for 30 min, in 50 ng/ml *C. difficile* toxin B for >6 h, or in $1 \mu\text{M}$ PMA for >24 h. On the other hand, the cells remained untreated (control), and bradykinin was applied either alone or together with $50 \mu\text{M}$ DEDA, which was also present for 5 min before the application of bradykinin; $n = 5$ to 9 ; ***, a significant difference versus control at $p < 0.001$; n.s., no significant difference.

led to a shape change and to detachment of the neurons after prolonged exposure (>7 h).

The inhibition of I_{Ca} in SCG neurons via M_1 muscarinic receptors involves phospholipase A_2 (PLA $_2$) (Liu et al., 2004), and this enzyme has also been reported to mediate cellular effects of bradykinin (Burch and Axelrod, 1987). However, the inhibition of I_{Ca} by bradykinin was not altered in the presence of 50 μ M DEDA (Fig. 3), which efficiently reduces the enzymatic activity of PLA $_2$ (Lister et al., 1989).

Recovery from the Inhibition of I_{Ca} Requires Lipid Kinase Activity or Intracellular PIP $_2$. Because none of the typical downstream effector systems of PLC- β seemed to be involved in the actions of bradykinin, we reasoned that a loss of membrane PIP $_2$ might be responsible for the reduction of I_{Ca} . If that was the case, PIP $_2$ resynthesis should be a prerequisite for the recovery from inhibition, and this requires ATP and lipid kinases, as demonstrated for the recovery of K_M channels from the inhibition via muscarinic receptors in SCG neurons (Suh and Hille, 2002; Zhang et al., 2003; Winks et al., 2005). Therefore, we investigated the effects of the lipid kinase inhibitors wortmannin (Nakanishi et al., 1995) and phenylarsine oxide (PAO) (Wiedemann et al., 1996) on the recovery from inhibition in perforated-patch recordings. When bradykinin was applied in the continuous presence of wortmannin (50 μ M) or PAO (30 μ M), I_{Ca} no longer recovered from the inhibition (Fig. 4, A, B, and E). To test for unspecific effects of PAO, this agent was incubated and applied together with 1 mM dithiothreitol (DTT), which traps PAO in stable inactive complexes (Schaefer et al.,

1994). DTT entirely prevented the inhibitory action of PAO on I_{Ca} recovery (Fig. 4, C and E). Besides inhibiting phosphatidylinositol 4-kinase (PI4-kinase), wortmannin also inhibits phosphatidylinositol 3-kinase (PI3-kinase) (Nakanishi et al., 1995). To verify that the loss of recovery in the presence of wortmannin was not caused by the inhibition of PI3-kinase, we investigated the effect of LY 294,002, a selective PI3-kinase inhibitor (Vlahos et al., 1994). Cultures were pretreated with 100 μ M LY 294,002 for 1 h, and then bradykinin was applied in the continuous presence of the inhibitor. Under these conditions, I_{Ca} displayed clear-cut recovery (Fig. 4, D and E). Because PAO was shown to inhibit PI4-kinase but not phosphatidylinositol 5-kinase activity (Wiedemann et al., 1998), the coincident inhibition of recovery by wortmannin and PAO but not by LY 294,002 suggests a major role for PI4-kinase.

The enzyme activity of PI4-kinase requires high intracellular ATP concentrations (Balla, 1998), and the recovery of K_M channels from the inhibition caused by PIP $_2$ depletion depended on the ATP concentration in the intracellular recording solution (Suh and Hille, 2002). When ATP in our pipette solution for whole-cell recordings was increased from the 2 mM standard concentration to 4 mM, the recovery of I_{Ca} from the bradykinin inhibition increased from 20% to >80% (Fig. 5A). The inner leaflet of the plasma membrane of SCG neurons had been calculated to contain 261 μ M PIP $_2$ under resting conditions (Winks et al., 2005). The addition of a similar concentration (200 μ M) of the more soluble diC8-PIP $_2$ to the whole-cell intracellular solution also led to a signifi-

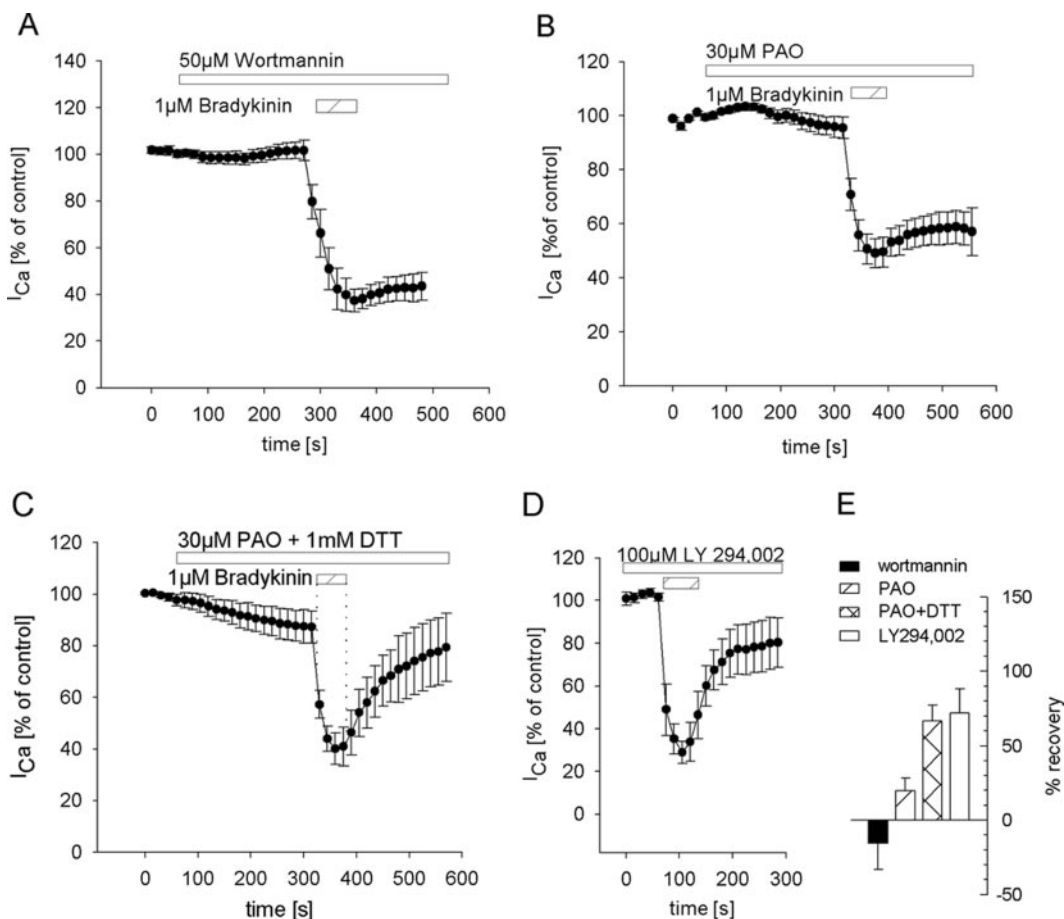


Fig. 4. Recovery of I_{Ca} from the inhibition by bradykinin in perforated-patch recordings: effects of lipid kinase inhibitors. Ca^{2+} currents were elicited by depolarizations from -80 to $+10$ mV once every 15 s. Bradykinin was present for 1-min periods, and the recovery of current amplitudes from the inhibition was calculated as shown in Fig. 2A. A, the time course of peak current amplitudes when 50 μ M wortmannin was applied before bradykinin ($n = 6$). B, the time course of peak current amplitudes when 30 μ M PAO was applied before bradykinin ($n = 5$). C, the time course of peak current amplitudes when 30 μ M PAO and 1 mM DTT was applied together before bradykinin ($n = 5$). D, the time course of peak current amplitudes when 100 μ M LY 294,002 was applied before bradykinin ($n = 5$). E, a summary of the extent of recovery under the aforementioned conditions.

cant increase in recovery (Fig. 5B). Thus, the supply of either PI4-kinase substrate or of the product of phosphatidylinositol-4-phosphate kinase is sufficient to re-establish the recovery of whole-cell I_{Ca} from the inhibition by bradykinin.

The above manipulations altered the recovery of I_{Ca} from the inhibition by bradykinin. In contrast, the extent of I_{Ca} inhibition caused by the peptide was not significantly changed under any of these conditions (Table 1). Hence, a continuous supply of PIP₂ to the membrane does not seem to be a prerequisite for the inhibition of I_{Ca} by bradykinin under the present electrophysiological recording conditions.

The Inhibition of [³H]Noradrenaline Release Involves PLC Activity and Changes in PIP₂. The data shown above indicate that PLC-dependent changes in PIP₂ mediate the inhibition of I_{Ca} by bradykinin. Because the inhibition of VACCs was the basis for the inhibition of noradrenaline release (Fig. 1), the same mechanisms should also be involved in the reduction of transmitter release by bradykinin. To test for this hypothesis, the inhibition of K⁺-evoked tritium efflux was investigated in SCG neurons loaded with [³H]noradrenaline and treated either with the PLC inhibitor U73122 (3 μ M) or with its inactive analog U73343 (3 μ M), both for 1 h. These two agents cannot be used during the determination of [³H]noradrenaline release because they cause large increases in spontaneous tritium outflow (Scholze et al., 2002). However, U73122 has been found to

block bradykinin effects in SCG neurons mediated by PLC in an irreversible manner (Bofill-Cardona et al., 2000). In U73122-treated cultures, bradykinin (1 μ M) failed to diminish the S2/S1 ratio of tritium efflux, but the peptide caused a significant inhibition in cultures treated with U73343 (Fig. 6A). For comparison, the inhibition of ³H efflux by the α_2 -adrenergic agonist UK 14304 (1 μ M) was also investigated in cultures treated with either U73122 (3 μ M) or its inactive analog. In that case, the results obtained after the two treatment procedures were not different from each other (Fig. 6A). Hence, only the presynaptic inhibition by bradykinin, but not that by an α_2 -adrenergic agonist, involved an activation of PLC.

To test for a role of PLC products in the inhibition of noradrenaline release by bradykinin, cultures were continuously superfused with 0.3 μ M thapsigargin, a concentration that abolishes the inhibition of K_M channels by the peptide (Bofill-Cardona et al., 2000). However, bradykinin reduced K⁺-evoked tritium efflux in the presence of thapsigargin to approximately the same extent as in its absence (Fig. 6B). We found previously that the inhibition of noradrenaline release by bradykinin was not attenuated when protein kinase C had been inhibited (Edelbauer et al., 2005). Hence, there was no evidence for a role of PLC products in the presynaptic inhibition caused by bradykinin.

To investigate whether changes in PIP₂ might be involved

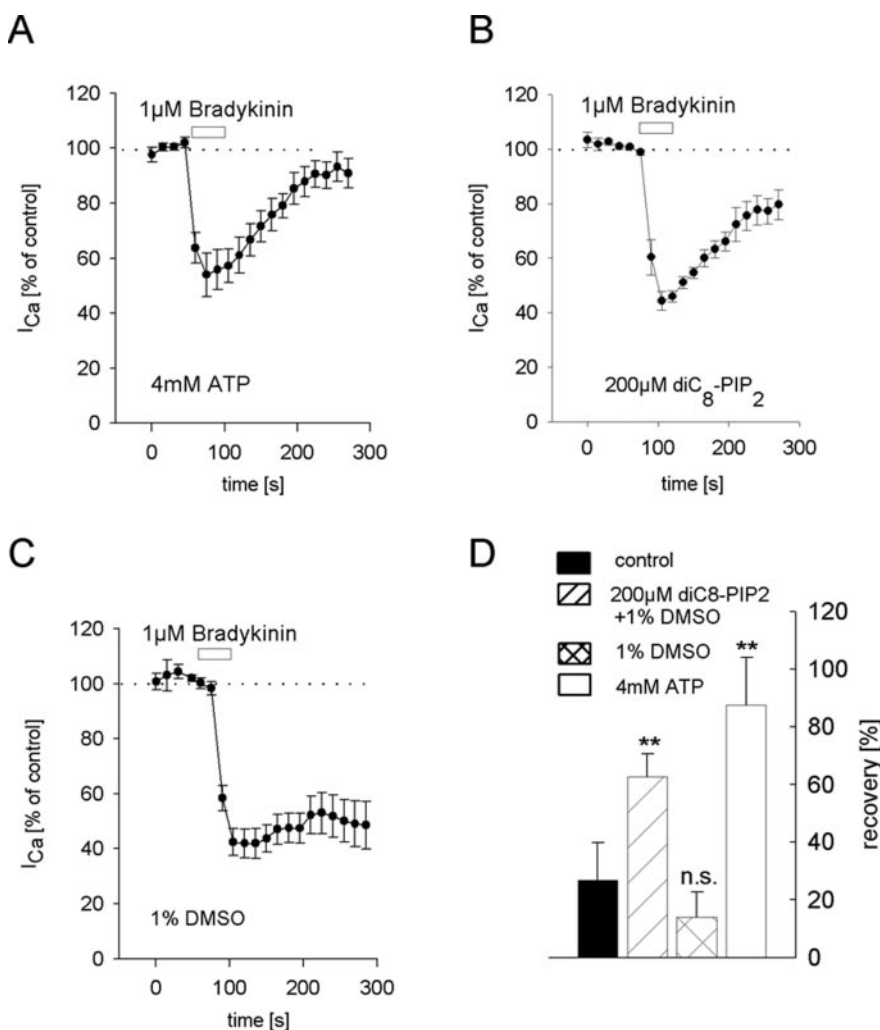


Fig. 5. Recovery of I_{Ca} from the inhibition by bradykinin in whole-cell recordings: effects of intracellular ATP and dioctanoyl PIP₂. Ca^{2+} currents were elicited by depolarizations from -80 to $+10$ mV once every 15 s. Bradykinin was present for 1-min periods, and the recovery of current amplitudes from the inhibition was calculated as shown in Fig. 2A. A, the time course of peak current amplitudes, with 4 mM ATP included in the pipette solution ($n = 8$). B, the time course of peak current amplitudes with 200 μ M dioctanoyl PIP₂ (diC8-PIP₂) and 1% DMSO included in the pipette solution ($n = 7$). C, the time course of peak current amplitudes with only 1% DMSO included in the pipette solution ($n = 5$). D, the extent of recovery under the aforementioned conditions and with the conventional whole-cell pipette solution (control; $n = 8$); **, a significant difference versus control at $p < 0.01$; n.s., no significant difference.

in the reduction of [^3H]noradrenaline release by bradykinin, PAO (10 μM) was applied before and during the second K^+ stimulation, either alone or together with bradykinin. PAO per se reduced the S2/S1 ratio of K^+ -evoked tritium efflux and prevented an additional inhibitory effect of bradykinin (Fig. 6C). The effects of PAO on I_{Ca} were prevented by DTT. Therefore, when PAO was applied together with 1 mM DTT, it neither caused a significant reduction of K^+ -evoked tritium efflux nor prevented the inhibitory action of bradykinin (Fig. 6C). Once again, UK 14304 (1 μM) was used instead of bradykinin for comparison, and the inhibitory effect of the

α_2 -adrenergic agonist turned out to be additive to that of PAO (Fig. 6C).

Discussion

In SCG and other postganglionic sympathetic neurons, a large number of GPCRs mediate presynaptic inhibition of noradrenaline release, on one hand, and a G protein-dependent inhibition of VACCs, on the other hand. All of the receptor subtypes that mediate both effects are linked to VACCs via a membrane-delimited interaction between G protein $\beta\gamma$ subunits and Ca^{2+} -channel proteins (Koh and Hille, 1997; Boehm and Kubista, 2002). Most recently, B_2 bradykinin receptors were added to this list of inhibitory presynaptic receptors (Edelbauer et al., 2005), and the present results demonstrate that the associated signaling cascade is a novel one and is definitely distinct from those described before.

Presynaptic P2X receptors of rat SCG neurons are highly Ca^{2+} permeable and thereby provide a route for transmembrane Ca^{2+} entry to trigger transmitter release that is independent of VACCs (Boehm, 1999). This type of stimulated noradrenaline release was altered neither by bradykinin nor by an α_2 -adrenergic agonist, but both agents did reduce noradrenaline release elicited by depolarizing K^+ concentrations. This confirms that α_2 -adrenergic receptors mediate a presynaptic inhibition of transmitter release from SCG neurons via an inhibition of VACCs (Boehm and Huck, 1995) and demonstrates that the same signaling mechanism is used by the inhibitory presynaptic B_2 receptors (Edelbauer et al.,

TABLE 1

Extent of the inhibition of I_{Ca} by bradykinin under conditions that alter the recovery from the inhibition

Experiments were performed as described in the legends to Figs. 4 and 5. For perforated-patch data, the p values are versus control; for whole-cell data, the p values are versus 2 mM ATP.

Condition	Inhibition	S.E.M.	n	p Values
	%			
Perforated patch				
Control	44.28	11.21	6	
50 μM Wortmannin	54.60	7.50	6	>0.29
30 μM PAO	48.33	3.89	5	>0.17
30 μM PAO + 1 mM DTT	54.80	4.63	5	>0.08
100 μM LY 294,002	68.85	5.09	5	>0.08
Whole cell				
2 mM ATP	49.92	3.53	6	
4 mM ATP	48.58	6.22	8	>0.94
200 μM diC8-PIP ₂	48.33	3.89	7	>0.64
1% DMSO	57.78	5.47	5	>0.23

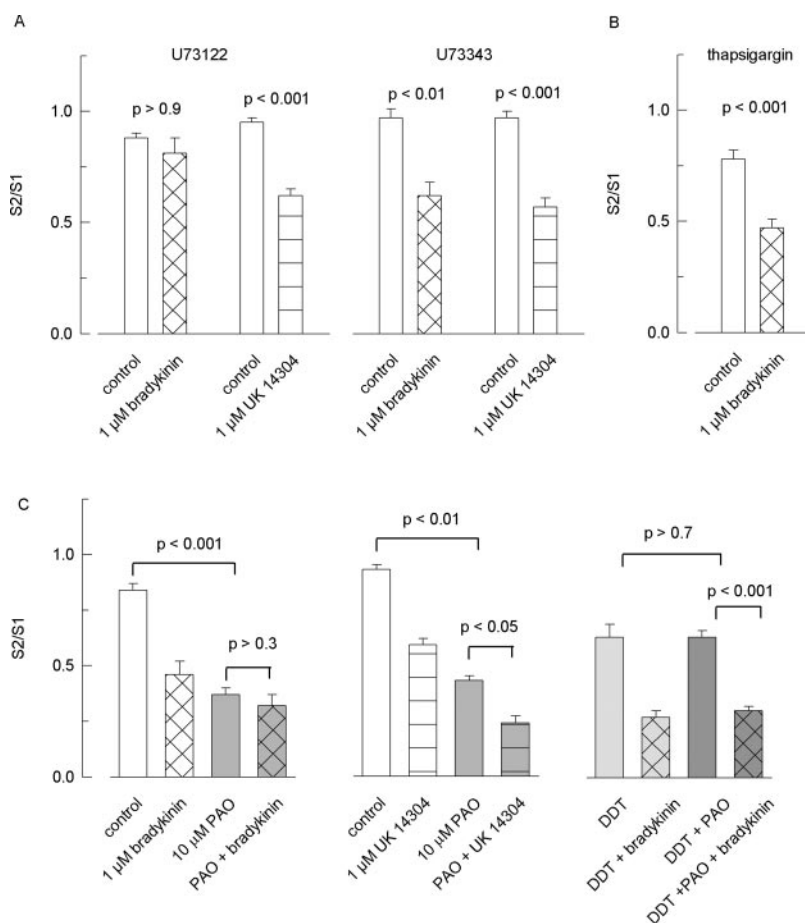


Fig. 6. Inhibition of K^+ -evoked noradrenaline release from SCG neurons by bradykinin: effects of PLC-, Ca^{2+} ATPase-, and lipid kinase inhibitors. After loading with [^3H]noradrenaline, neurons were superfused, and subsequent to a 1-h washout period, 4-min fractions of superfusate were collected. After 68 (S1) and 88 (S2) min of superfusion, the cultures were stimulated for 60 s by the presence of 40 mM K^+ (Na^+ was reduced accordingly). Results are shown as S2/S1 ratios of stimulated tritium efflux. A, cultures had been treated with either 3 μM U73122 or 3 μM U73343 during the [^3H]noradrenaline-uptake period. Bradykinin (1 μM) or UK 14304 (1 μM) was present from minute 86 onward, as shown in Fig. 1A, or no receptor agonist was added to the superfusion buffer (control); $n = 7$ to 9. B, thapsigargin (0.3 μM) was present from minute 50 of superfusion onward. Bradykinin (1 μM) was present from minute 86 onward, as shown in Fig. 1A, or no receptor agonist was added to the superfusion buffer (control); $n = 12$. C, bradykinin (1 μM) or UK 14304 (1 μM) was present for 2 min, and PAO (10 μM) and dithiothreitol (1 mM) for 4 min, before and during the second K^+ stimulation period, either alone or in combination. On the other hand, no drug was added to the superfusion buffer (control); $n = 8$ to 9.

2005). Nevertheless, the signal cascade that linked B₂ receptors to VACCs was clearly different from that of α_2 -adrenoceptors: the reduction of I_{Ca} by bradykinin was not altered by large depolarizing prepulses nor by a treatment of the neurons with pertussis toxin, two manipulations that attenuated or abolished the reduction of I_{Ca} by an α_2 -adrenergic agonist. Hence, bradykinin controls VACCs via mechanisms different from those of α_2 -adrenoceptors which do so through a direct interaction of G protein $\beta\gamma$ subunits with channel proteins (Ikeda and Dunlap, 1999; Elmslie et al., 2003).

In sympathetic neurons, bradykinin inhibits not only VACCs, as described here, but also K_M channels (Jones et al., 1995). This latter effect involves PLC-dependent decreases in membrane PIP₂ and IP₃-dependent increases in intracellular Ca²⁺ (Cruzblanca et al., 1998; Bofill-Cardona et al., 2000; Winks et al., 2005). Only the former mechanism, the decrease in membrane PIP₂, was responsible for the bradykinin-dependent inhibition of VACCs, as revealed by the following results: 1) the PLC inhibitor U73122 almost abolished the inhibition of I_{Ca} , but the inactive analog U73343 had no effect; 2) the depletion of intracellular Ca²⁺ stores by thapsigargin or the chelation of intracellular Ca²⁺ ions by BAPTA failed to alter the bradykinin inhibition of I_{Ca} . Nevertheless, Ca²⁺ influx via VACCs was required for an efficient inhibition of the channels, because Ba²⁺ currents were less affected by the peptide than Ca²⁺ currents; 3) the inhibition of protein kinase C, whether by GF 109203X or by long-term phorbol ester treatment, did not attenuate the inhibitory effect of bradykinin on I_{Ca} ; 4) the inactivation of Rho proteins, which mediate the bradykinin inhibition of VACCs in neuroblastoma cells (Wilk-Blaszczak et al., 1997), did not affect the reduction of I_{Ca} in SCG neurons; and 5) the inhibition of PLA₂ by DEDA failed to alter the I_{Ca} reduction by bradykinin, although this enzyme is involved in the modulation of VACCs in SCG neurons via M₁ muscarinic acetylcholine receptors (Liu et al., 2004). Taken together, bradykinin inhibited I_{Ca} via PLC, but it did so independently of downstream effectors or alternative signaling cascades.

The reduction of membrane PIP₂ by bradykinin has been demonstrated most recently in SCG neurons (Gamper et al., 2004; Winks et al., 2005). The kinetics of this effect have been studied in detail in neuroblastoma cells (Xu et al., 2003). There, the peptide caused a short (<10 s) transient increase in PIP₂ and a subsequent decrease that was maximal after 30 s to 1 min. This was followed by a slow resynthesis that brought PIP₂ levels back to control within up to 3 min. This time course of PIP₂ depletion was much slower than the increase in IP₃, which was maximal within less than 10 s (Xu et al., 2003). Hence, the kinetics of the bradykinin-induced changes in membrane PIP₂, but not the changes in PLC products, paralleled the time course of the inhibition of I_{Ca} as observed here. In SCG neurons, the concentration of membrane PIP₂ is directly correlated with the conductance of K_M channels (Winks et al., 2005), and the time course of K_M channel inhibition by bradykinin (Jones et al., 1995) is almost congruent with the present time course of I_{Ca} inhibition. Recombinant VACCs, in particular P/Q- (Wu et al., 2002) and N-type (Gamper et al., 2004) channels, were also found to be regulated by the membrane PIP₂ concentration. Sympathetic neurons express N-type, rather than P/Q-type, Ca²⁺ channels, and the inhibition of I_{Ca} in sympathetic neurons by luteinizing hormone-releasing hormone was reported

to be PLC-dependent (Wu et al., 2002). In addition, evidence has been presented that activation of M₁ muscarinic receptors inhibited VACCs in rat SCG neurons through a depletion of membrane PIP₂ (Gamper et al., 2004). Moreover, bradykinin was found to enhance currents through VR₁ receptors via a depletion of membrane PIP₂ (Chuang et al., 2001). Taken together, there is experimental evidence showing that bradykinin receptors control the membrane levels of PIP₂ and that a number of ion channels, including N-type VACCs, are regulated by PIP₂. In the present experiments, the recovery of I_{Ca} from the inhibition by bradykinin required replenishment of membrane PIP₂, as evidenced by the following results: 1) in whole-cell recordings, high intracellular ATP concentrations were required for full recovery of I_{Ca} , and they are necessary for maximal PI4-kinase activity (Balla, 1998); 2) independently of the ATP concentration, the addition of diC8-PIP₂ to the whole-cell intracellular solution enabled the recovery of I_{Ca} ; and 3) inhibition of PI4-kinase activity by wortmannin or PAO prevented the recovery of I_{Ca} from the inhibition by bradykinin, which was otherwise seen in perforated-patch recordings. Thus, together with the previous observations, the present results indicate that a PLC-induced reduction of membrane PIP₂ mediates the bradykinin inhibition of VACCs.

Bradykinin was recently reported to inhibit VACCs in SCG neurons only when the resynthesis of PIP₂ was blocked (Gamper et al., 2004). In the present experiments, however, the peptide reduced I_{Ca} whether lipid kinases were inhibited or not, and the PI4-kinase inhibitors affected only the recovery of I_{Ca} , but not the extent of inhibition elicited by bradykinin. Although these results are somehow contradictory, they both do confirm that VACCs are controlled by the relation of PIP₂ catalysis and synthesis. Nevertheless, it remains elusive why the efficiency of bradykinin receptors in mediating the depletion of membrane PIP₂ may differ. Causative factors for the observed discrepancies might be 1) differences in the composition of culture media and sera because growth factors also control membrane PIP₂ (Chuang et al., 2001); 2) differences in the buffering of the intracellular Ca²⁺ concentration as the PIP₂ synthesis may be altered by changes in intracellular Ca²⁺ (Gamper et al., 2004; Winks et al., 2005); 3) differences in intracellular Mg²⁺, millimolar concentrations of which are required for maximal G protein signaling (Suh et al., 2004); and 4) differences in the voltage protocols used to elicit I_{Ca} because repeated depolarizations are known to reduce membrane PIP₂ (Micheva et al., 2001).

The inhibition of VACCs was found to be a prerequisite for the reduction of transmitter release by bradykinin. Therefore, the PLC-mediated depletion of membrane PIP₂, as involved in the inhibition of I_{Ca} at neuronal somata, should also play a role in the presynaptic inhibition. In accordance with this expectation, the active PLC inhibitor U73122 abolished the reduction of noradrenaline release by bradykinin without affecting the inhibitory action of the α_2 agonist. However, inhibition of protein kinase C (Edelbauer et al., 2005) or depletion of intracellular Ca²⁺ stores by thapsigargin left the inhibition of noradrenaline release by bradykinin unaltered. In contrast, inhibition of lipid kinases by PAO reduced noradrenaline release and prevented a further reduction by bradykinin. The α_2 -adrenergic agonist, however, did reduce release even in the presence of PAO. All of the effects of PAO on noradrenaline release were abolished by DTT, which also

prevents the inhibition of lipid kinases (Schaefer et al., 1994). These results indicate that not the products of PLC but rather changes in the substrate, PIP_2 , are involved in the presynaptic inhibition by bradykinin. Nevertheless, these results are not congruent with those obtained for the inhibition of I_{Ca} : PAO did reduce noradrenaline release and prevented a further reduction by bradykinin, two effects not observed with I_{Ca} . Several facts may explain these apparent discrepancies. First, lipid kinases that are inhibited by PAO are associated not only with the plasma membrane (Micheva et al., 2001) but also with vesicle membranes (Wiedemann et al., 1996). Therefore, the inhibition of both pools of enzymes may contribute to the inhibition of release, but only one pool can be involved in the regulation of I_{Ca} . Second, repeated or prolonged depolarizing stimuli are known to strongly reduce the PIP_2 contents of presynaptic membranes, and its resynthesis is blocked by PAO (Micheva et al., 2001). Therefore, our 1-min K^+ stimulation to trigger noradrenaline release will decrease PIP_2 in the presynaptic membrane, but the 30-ms depolarizations used to evoke I_{Ca} are unlikely to do so. As a consequence, bradykinin may be able to reduce I_{Ca} through PIP_2 depletion in current recordings from neuronal somata but fails to further reduce the PIP_2 associated with presynaptic VACCs during the stimulation of noradrenaline release. Third, VACCs are highly concentrated at the sites of vesicle exocytosis (Stevens, 2004), and there they are clustered in lipid microdomains (Taverna et al., 2004). It thus seems reasonable to assume that the quantitative relation between membrane PIP_2 and VACCs is different between neuronal somata and presynaptic nerve terminals. Nevertheless, in both locations, bradykinin controls the function of VACCs through PLC-dependent changes in PIP_2 , as indicated by the present results. In conclusion, our results show that bradykinin inhibits VACCs of sympathetic neurons through a PLC-mediated depletion of membrane PIP_2 and demonstrate that this effect provides a novel mechanism for the presynaptic inhibition of transmitter release via GPCRs.

References

- Balla T (1998) Phosphatidylinositol 4-kinases. *Biochim Biophys Acta* **1436**:69–85.
- Boehm S (1999) ATP stimulates sympathetic transmitter release via presynaptic P_2X purinoceptors. *J Neurosci* **19**:737–746.
- Boehm S and Huck S (1995) α_2 -adrenoreceptor-mediated inhibition of acetylcholine-induced noradrenaline release from rat sympathetic neurons: an action at voltage-gated Ca^{2+} channels. *Neuroscience* **69**:221–231.
- Boehm S and Huck S (1997) Noradrenaline release from rat sympathetic neurones triggered by activation of B_2 bradykinin receptors. *Br J Pharmacol* **122**:455–462.
- Boehm S, Huck S, and Freissmuth M (1996) Involvement of a phorbol ester-insensitive protein kinase C in the α_2 -adrenergic inhibition of voltage-gated calcium current in chick sympathetic neurons. *J Neurosci* **16**:4596–4603.
- Boehm S and Kubista H (2002) Fine tuning of sympathetic transmitter release via ionotropic and metabotropic presynaptic receptors. *Pharmacol Rev* **54**:43–99.
- Bofill-Cardona E, Vartian N, Nanoff C, Freissmuth M, and Boehm S (2000) Two different signaling mechanisms involved in the excitation of rat sympathetic neurons by uridine nucleotides. *Mol Pharmacol* **57**:1165–1172.
- Burch RM and Axelrod J (1987) Dissociation of bradykinin-induced prostaglandin formation from phosphatidylinositol turnover in Swiss 3T3 fibroblasts: evidence for G protein regulation of phospholipase A2. *Proc Natl Acad Sci USA* **84**:6374–6378.
- Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, and Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from $\text{PtdIns}(4,5)\text{P}_2$ -mediated inhibition. *Nature (Lond)* **411**:957–962.
- Cruzblanca H, Koh DS, and 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.
- Edelbauer H, Lechner SG, Mayer M, Scholze T, and Boehm S (2005) Presynaptic inhibition of transmitter release from rat sympathetic neurons by bradykinin. *J Neurochem* **93**:1110–1121.
- Elmslie KS (2003) Neurotransmitter modulation of neuronal calcium channels. *J Bioenerg Biomembr* **35**:477–489.
- Filippov AK, Simon J, Barnard EA, and Brown DA (2003) Coupling of the nucleotide P_2Y_4 receptor to neuronal ion channels. *Br J Pharmacol* **138**:400–406.
- Foucart S, Gibbons SJ, Brorson JR, and Miller R (1995) Increases in $[\text{Ca}^{2+}]_i$ by CCh in adult rat sympathetic neurons are not dependent on intracellular Ca^{2+} pools. *Am J Physiol* **268**:C829–C837.
- Gamper N, Reznikov V, Yamada Y, Yang J, and Shapiro MS (2004) Phosphatidylinositol 4,5-bisphosphate signals underlie receptor-specific $\text{Gq}/11$ -mediated modulation of N-type Ca^{2+} channels. *J Neurosci* **24**:10980–10992.
- Haley JE, Abogadie FC, Fernandez-Fernandez JM, Dayrell M, Vallis Y, Buckley NJ, and Brown DA (2000) Bradykinin, but not muscarinic, inhibition of M-current in rat sympathetic ganglion neurons involves phospholipase C- β 4. *J Neurosci* **20**:RC105.
- Hille B (1994) Modulation of ion channel function by G protein-coupled receptors. *Trends Neurosci* **17**:531–535.
- Ikedo SR and Dunlap K (1999) Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. *Adv Second Messenger Phosphoprotein Res* **33**:131–151.
- Jones S, Brown DA, Milligan G, Willer E, Buckley NJ, and Caulfield MP (1995) Bradykinin excites rat sympathetic neurons by inhibition of M current through a mechanism involving B_2 receptors and $\text{Gq}/11$. *Neuron* **14**:400–405.
- Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M, and Aktories K (1995) Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature (Lond)* **375**:500–503.
- Koh DS and Hille B (1997) Modulation by neurotransmitters of catecholamine secretion from sympathetic ganglion neurons detected by amperometry. *Proc Natl Acad Sci USA* **94**:1506–1511.
- Lister MD, Glaser KB, Ulevitch RJ, and Dennis EA (1989) Inhibition studies on the membrane-associated phospholipase A2 in vitro and prostaglandin E2 production in vivo of the macrophage-like P388D1 cell. Effects of manoolide, 7,7-dimethyl-5,8-eicosadienoic acid and p-bromophenacyl bromide. *J Biol Chem* **264**:8520–8528.
- Liu L, Roberts ML, and Rittenhouse AR (2004) Phospholipid metabolism is required for M1 muscarinic inhibition of N-type calcium current in sympathetic neurons. *Eur Biophys J* **33**:255–264.
- Micheva KD, Holz RW, and Smith SJ (2001) Regulation of presynaptic phosphatidylinositol 4,5-bisphosphate by neuronal activity. *J Cell Biol* **154**:355–368.
- Nakanishi S, Catt KJ, and Balla T (1995) A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. *Proc Natl Acad Sci USA* **92**:5317–5321.
- Schaefer T, Wiedemann C, Gitler C, and Burger MM (1994) Effects of arsenicals on the secretory process in chromaffin cells. *Ann NY Acad Sci* **710**:356–367.
- Scholze T, Moskvina E, Mayer M, Just H, Kubista H, and Boehm S (2002) Sympathoexcitation by bradykinin involves Ca^{2+} -independent protein kinase C. *J Neurosci* **22**:5823–5832.
- Schwartz DD and Malik KU (1993) Cyclic AMP modulates but does not mediate the inhibition of $[\text{H}]$ norepinephrine release by activation of α -2 adrenergic receptors in cultured rat ganglion cells. *Neuroscience* **52**:107–113.
- Stevens CF (2004) Presynaptic function. *Curr Opin Neurobiol* **14**:341–345.
- Suh BC and Hille B (2002) Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron* **35**:507–520.
- Suh BC, Horowitz LF, Hirdes W, Mackie K, and Hille B (2004) Regulation of KCNQ2/KCNQ3 current by G protein cycling: the kinetics of receptor-mediated signaling by Gq . *J Gen Physiol* **123**:663–683.
- Taverna E, Saba E, Rowe J, Francolini M, Clementi F, and Rosa P (2004) Role of lipid microdomains in P/Q-type calcium channel (Cav2.1) clustering and function in presynaptic membranes. *J Biol Chem* **279**:5127–5134.
- Vlahos CJ, Matter WF, Hui KY, and Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY 294,002). *J Biol Chem* **269**:5241–5248.
- Wiedemann C, Schafer T, and Burger MM (1996) Chromaffin granule-associated phosphatidylinositol 4-kinase activity is required for stimulated secretion. *EMBO (Eur Mol Biol Organ) J* **15**:2094–2101.
- Wiedemann C, Schafer T, Burger MM, and Sihra TS (1998) An essential role for a small synaptic vesicle-associated phosphatidylinositol 4-kinase in neurotransmitter release. *J Neurosci* **18**:5594–5602.
- Wilk-Blaszczak MA, Singer WD, Quill T, Miller B, Frost JA, Sternweis PC, and Belardetti F (1997) The monomeric G-proteins Rac1 and/or Cdc42 are required for the inhibition of voltage-dependent calcium current by bradykinin. *J Neurosci* **17**:4094–4100.
- Winks JS, Hughes S, Filippov AK, Tatunian L, Abogadie FC, Brown DA, and Marsh SJ (2005) Relationship between membrane phosphatidylinositol 4,5-bisphosphate and receptor-mediated inhibition of native neuronal M channels. *J Neurosci* **25**:3400–3413.
- Wu L, Bauer CS, Zhen XG, Xie C, and Yang J (2002) Dual regulation of voltage-gated calcium channels by $\text{PtdIns}(4,5)\text{P}_2$. *Nature (Lond)* **419**:947–952.
- Xu C, Watras J, and Loew LM (2003) Kinetic analysis of receptor-activated phosphoinositide turnover. *J Cell Biol* **161**:779–791.
- Zhang H, Craciun LC, Mirshahi T, Rohacs T, Lopes CM, Jin T, and Logothetis DE (2003) PIP_2 activates KCNQ channels and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron* **37**:963–975.

Address correspondence to: Dr. Stefan Boehm, Department of Pharmacology, Center of Biomolecular, Medicine and Pharmacology, Medical University of Vienna, Waehringerstrasse 13a, A-1090 Vienna, Austria. E-mail: Stefan.Boehm@meduniwien.ac.at