Inhibition of voltage-dependent sodium channels by Ro 31-8220, a 'specific' protein kinase C inhibitor[1]

Ratnakumari Lingameneni[a], Tanya N. Vysotskaya[a], Daniel S. Duch[a,b], Hugh C. Hemmings Jr.[a,c,*]

[a] *Department of Anesthesiology, Weill Medical College of Cornell University, Box 50, LC-203A, 525 East 68th Street, New York, NY 10021, USA*
[b] *Department of Physiology and Biophysics, Weill Medical College of Cornell University, 525 East 68th Street, New York, NY 10021, USA*
[c] *Department of Pharmacology, Weill Medical College of Cornell University, 525 East 68th Street, New York, NY 10021, USA*

Received 20 March 2000

Edited by Maurice Montal

Abstract We find that several protein kinase C (PKC) inhibitors, previously considered to be specific, directly inhibit voltage-dependent Na^+ channels at their useful concentrations. Bisindolylmaleimide I (GF 1092037), IX (Ro 31-8220) and V (an inactive analogue), but not H7 (a non-selective isoquinolinesulfonamide protein kinase inhibitor), inhibited Na^+ channels assessed by several independent criteria: Na^+ channel-dependent glutamate release and [^{3}H]batrachotoxinin-A 20-α-benzoate binding in rat cortical synaptosomes, veratridine-stimulated $^{22}Na^+$ influx in CHO cells expressing rat CNaIIa Na^+ channels and Na^+ currents measured in isolated rat dorsal root ganglion neurons by whole cell patch-clamp recording. These findings limit the usefulness of the bisindolylmaleimide class PKC inhibitors in excitable cells.

Key words: Protein kinase inhibitor; Protein kinase C; Glutamate release; Na^+ channel; Synaptosome; Dorsal root ganglion neuron

1. Introduction

Specific inhibitors of protein kinases are useful probes for analyzing signal transduction pathways, but their usefulness in intact cells can be limited by non-specific actions on other targets [1]. The bisindolylmaleimide derivatives of staurosporine are widely used as specific inhibitors of conventional protein kinase C (PKC) isoforms. The specificity of inhibitors such as these, which act as competitive inhibitors of ATP binding to the nucleotide binding site of PKC [2], is limited by the conserved protein kinase catalytic domain of most protein-serine/threonine kinases [3]. This limitation is evident in recent reports identifying inhibition by bisindolylmaleimides of other protein kinases, including glycogen synthase kinase-3 [4], mitogen-activated protein kinase-activated protein kinase-1β (Rsk-2) and p70 S6 kinase [5], with potencies greater than or comparable to inhibition of PKC (IC_{50} values in the nM range). Effects of bisindolylmaleimides on targets other than protein kinases have also been identified recently, including inhibition of 5-hydroxytryptamine$_3$ receptors with a potency comparable to inhibition of PKC [6], and of chromaffin cell nicotinic receptors [7] and human muscarinic receptors [8] at higher concentrations. Here we report that bisindolylmaleimides I (GF 109203X), IX (Ro 31-8220) and V (inactive against PKC), but not H7 (a non-selective isoquinolinesulfonamide protein kinase inhibitor), inhibit Na^+ channels at low micromolar concentrations in rat cortical synaptosomes and dorsal root ganglion (DRG) neurons by several criteria. These 'non-specific' actions place considerable restrictions on the use of these compounds as PKC inhibitors in intact cells, particularly in excitable cells.

2. Materials and methods

Experiments were done in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals as approved by the Weill Medical College of Cornell University Institutional Animal Care and Use Committee.

2.1. Materials

Bisindolylmaleimides I (GF 109203X), IX (Ro 31-8220) and V were obtained from Calbiochem (La Jolla, CA, USA); $NADP^+$, L-glutamate dehydrogenase (*Proteus* sp.), bovine serum albumin (BSA; essentially fatty acid free), 4-aminopyridine (4AP), veratridine, tetrodotoxin (TTX), scorpion toxin, dimethyl sulfoxide (DMSO), collagenase (type IV), trypsin (type XII), pronase E (type XIV) and H7 were from Sigma (St. Louis, MO, USA); Percoll was from Pharmacia (Uppsala, Sweden); Hanks balanced salt solution, fetal bovine serum, D-MEM/F-12, penicillin and streptomycin were from Gibco BRL (Gaithersburg, MD, USA) and [^{3}H]batrachotoxinin-A 20-α-benzoate ([^{3}H]BTX-B; 34 Ci/mmol) and ^{22}NaCl (1 mCi/ml) were from DuPont-New England Nuclear (Boston, MA, USA). All other chemicals were of analytical grade.

2.2. Isolation of synaptosomes

Synaptosomes were prepared from the cerebral cortex of adult Sprague–Dawley rats by the method of Dunkley et al. [9] with minor modifications [10]. Purified synaptosomes were suspended in HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) buffered medium (HBM; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 5 mM $NaHCO_3$, 10 mM D-glucose and 20 mM HEPES, pH 7.4 with NaOH), pelleted by centrifugation at 8000×*g* for 10 min and stored on ice for up to 5 h until use.

2.3. Measurement of glutamate release

The release of endogenous glutamate was measured by an enzyme-linked fluorescence method [11]. Synaptosomal pellets (0.5 mg protein) were resuspended in 1.5 ml HBM plus 16 μM BSA, 1 mM $NADP^+$, 100 U L-glutamate dehydrogenase, and 1.3 mM $CaCl_2$. Stirred samples were equilibrated at 37°C for 4 min in a spectrofluorometer cuvette and data acquisition was started (excitation wavelength = 340 nm; emission wavelength = 460 nm). After recording basal fluorescence change (ΔF) for 60 s, inhibitors (1.5–3.0 μl in DMSO) were added and ΔF was measured from 0–60 s. 200 s after the addition of inhibitor, secretagogue (6 μl of 0.25 M 4AP, 3 μl of

*Corresponding author. Fax: (1)-212-746 8316.
E-mail: hchemmi@mail.med.cornell.edu

[1] Presented in part at the 28th Annual Meeting of the Society for Neuroscience (November 7–12, 1998, Los Angeles, CA, USA).

PII: S0014-5793(00)01532-5

10 mM veratridine, 15 μl of 3 M KCl or 3.0 μl of 25 mM monensin) was added and the initial ΔF (0–60 s) was measured. The fluorescence signal was calibrated by adding 5 nmol of L-glutamate to the cuvette at the end of each experiment.

Glutamate release evoked by 4AP, veratridine or KCl was measured to assess the involvement of presynaptic Na^+ channels as targets for drug actions. 4AP, a K^+ channel blocker, destabilizes membrane potential and causes repetitive spontaneous Na^+ channel-dependent (tetrodotoxin-sensitive) depolarizations which mimic depolarization of nerve terminals by action potentials and lead to activation of Ca^{2+} channels and neurotransmitter release. Veratridine activates Na^+ channels directly and thereby depolarizes the plasma membrane leading to Ca^{2+} channel activation and Na^+ channel-dependent (tetrodotoxin-sensitive) neurotransmitter release. Elevated extracellular KCl concentrations depolarize the plasma membrane by shifting the K^+ equilibrium potential above the threshold potential for activation of Ca^{2+} channels, leading to Ca^{2+} entry and Na^+ channel-independent (tetrodotoxin-insensitive) neurotransmitter release, while Na^+ channels are inactivated [12].

2.4. Measurement of [^{3}H]BTX-B binding and $^{22}Na^+$ influx

Equilibrium binding of 10 nM [^{3}H]BTX-B to intact synaptosomes in the presence of scorpion toxin (*Leirus quinquestriatus*) and veratridine (60 μM)-stimulated Na^+ influx into CHO CNaIIa cells was determined as described [13].

2.5. Isolation of DRG neurons

Intermediate sized DRG neurons (15–40 μm diameter) were isolated from Sprague–Dawley rats aged 5–30 days by modification of described methods [14,15]. Briefly, animals were anesthetized with 80% CO_2/20% O_2, decapitated and DRG were quickly removed and placed in oxygenated, ice-cold normal or Ca^{2+}-free Hanks balanced salt solution (5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 137.9 mM NaCl, 0.3 mM Na_2HPO_4 and 5.6 mM glucose ± 1.3 mM $CaCl_2$) containing one or a combination (for control purposes) of the following proteases (in w/v): trypsin (0.1–0.2%), collagenase (0.1%) or Pronase E (0.05–1%). The different combination of proteases or the age of animals did not affect the maximum Na^+ current and/or drug effects. After enzyme incubation, ganglia were placed in D-MEM/F-12 containing 20% (v/v) fetal bovine serum and triturated with a Pasteur pipette. Dissociated cells were plated on polylysine covered Petri dishes. Cells were used within a few hours of dissociation and for up to 3 days of primary culture in 95% O_2/5% CO_2 at 37°C. The culture medium consisted of D-MEM/F-12 with L-glutamine, 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.6. Electrophysiology

Na^+ currents in DRG neurons were studied by whole-cell patch-clamp recording using a standard patch-clamp amplifier (Axopatch 200, Axon Instruments, Foster City, CA, USA) that was controlled by commercially available software (pCLAMP, Axon Instruments). Patch-clamp pipettes were pulled from micropipette glass (Drummond, Broomall, PA, USA) and filled with intracellular solution (10 mM NaCl, 140 mM CsF, 5 mM HEPES, pH 7.2). The external solution was Hanks balanced salt solution plus 20 mM tetraethylammonium chloride, 5 mM HEPES and 5 μM $LaCl_3$, pH 7.2. Under these conditions, TTX-resistant channels, which are present in some DRG neurons, were blocked [16], and only currents through TTX-sensitive channels were recorded. This was verified by recordings in the presence of TTX (1 μM), which blocked all Na^+ currents (data not shown). Drugs were applied via a glass–PTFE perfusion system with a superfusion pipette (flow rate 0.5–0.8 ml/min) close to the cell. Recordings were made at room temperature (22 ± 1°C). Currents were filtered at 5 kHz, digitized and recorded to hard disk. Capacitive transients and series resistance were measured and compensated using the amplifier's internal compensation circuitry; active series resistance compensation was used to compensate 60–85% of the series resistance. Cells with currents greater than 6 nA or less than 1 nA, or with leakage greater than 5% of maximal Na^+ conductance were rejected.

To estimate the application kinetics, we routinely examined currents with a test pulse to +10 mV from a holding potential of −70 mV. The voltage protocol used to measure current–voltage (I–V) relationships consisted of a preconditioning step to −100 mV and test pulses from a holding potential of −70 mV to test potentials of −60 to +70 mV. The voltage protocol used to measure steady-state inactivation consisted of prepulses to varying potentials (500 ms to potentials between −150 and −10 mV) and a test pulse to +10 mV.

2.7. Data analysis

Statistical differences between control and experimental values were determined by analysis of variance (ANOVA) with Fisher's post hoc test or Student's t-test as appropriate. Concentration–effect data were analyzed for IC_{50} values by linear regression using data between 20 and 80% of the maximal response (Pharm/PCS Pharmacologic Calculation System, Version 4.2, Springer, New York, NY, USA). Values are expressed as mean ± S.D. Electrophysiological analysis was performed on-line with a second acquisition computer using pCLAMP6. Curve fits and statistical analysis were performed using commercially available software: Origin and pCLAMP Module for Origin (Microcal Software, Northampton, MA, USA).

2.8. Miscellaneous

Protein concentrations were determined by the Coomassie blue method [17] using BSA as a standard.

3. Results

3.1. Effects of Ro 31-8220 on rat cortical synaptosomal Na^+ channels

Ro 31-8220 inhibited glutamate release evoked by 1 mM 4AP or 20 μM veratridine, but not by 30 mM KCl or 50 μM monensin (Fig. 1A). A similar pattern of inhibition was observed for 1 μM TTX (data not shown), suggesting the involvement of Na^+ channels in the Ro 31-8220 effect. This possibility was supported by the observations that Ro 31-8220 potently inhibited [^{3}H]BTX binding to synaptosomes (IC_{50} = 1.1 μM; Fig. 1B) by a competitive mechanism (data not shown). Ro 31-8220 also inhibited veratridine-stimulated

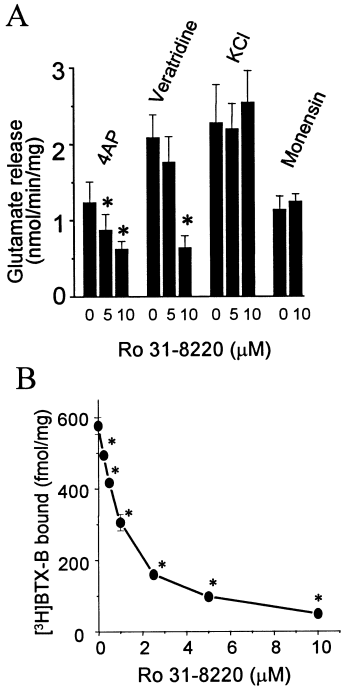

Fig. 1. Effects of Ro 31-8220 on synaptosomal Na^+ channels. A: Ro 31-8220 inhibited Na^+ channel-dependent glutamate release evoked by 4-aminopyridine and veratridine, but not Na^+ channel-independent glutamate release evoked by elevated KCl or monensin. B: Ro 31-8220 inhibited specific [^{3}H]BTX-B binding to synaptosomal Na^+ channels. Summary data expressed as mean ± S.D. (n = 3). *P < 0.05 versus control (no Ro 31-8220) by ANOVA with Fisher's post hoc test.

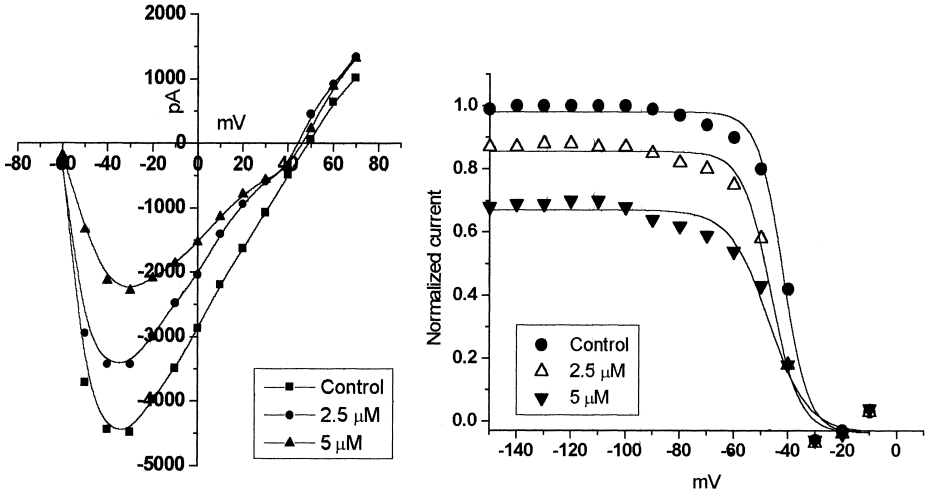

Fig. 2. Effects of Ro 31-8220 on whole-cell Na^+ current in isolated DRG neurons. Left: Representative peak current–voltage relationships are shown for currents in the presence and absence of Ro 31-8220, elicited by voltage steps from a conditioning potential of −100 mV to test potentials varying from −60 to +100 mV in two separate cells. Right: Effect of Ro 31-8220 on Na^+ channel steady-state inactivation. Na^+ currents were elicited by test pulses to −10 mV after 500 ms pre-pulses to potentials varying from −150 to −10 mV. Representative peak currents were normalized and plotted as a function of pre-pulse potential.

$^{22}Na^+$ influx into CHO cells expressing rat CNaIIa Na^+ channels (10.2 ± 0.79 (control) versus 7.26 ± 1.25 (3.3 μM Ro 31-8220) nmol/min/mg protein; $P < 0.05$, $n = 3$). Bisindolylmaleimide I (GF 109203X; 5–20 μM) also significantly inhibited glutamate release evoked by veratridine (24, 39 and 85% inhibition by 5, 10 and 20 μM, respectively), but not by elevated KCl, while the non-selective isoquinolinesulfonamide protein kinase inhibitor H7 (100 μM) did not significantly affect either (data not shown).

3.2. Effects of PKC inhibitors on DRG neuronal Na^+ currents

Ro 31-8220 significantly inhibited peak Na^+ currents in isolated rat DRG neurons in a concentration-dependent manner (Fig. 2, left). Significant effects were observed within 20 s of solution exchange, maximal effects were observed within 2 min of application and no further changes in Na^+ current were observed for 20–40 min, depending on the drug. Therefore, drug effects were measured after 2 min of application, when compounds had reached an apparent steady-state. There was no significant effect on the voltage-dependence of inactivation (Fig. 2, right). In contrast, H7 had no significant effect on DRG Na^+ currents (Fig. 3). Bisindolylmaleimide I (GF 109203X) and bisindolylmaleimide V (inactive against PKC), which are structurally related to Ro 31-8220 but differ in their activity as PKC inhibitors, both significantly inhibited peak Na^+ currents, but with slightly lower potencies (Fig. 3).

4. Discussion

Our results demonstrate that the bisindolylmaleimides Ro 31-8220 and GF 109203X, among the most commonly used PKC inhibitors [1,2,18], inhibit voltage-dependent Na^+ channels at concentrations commonly employed in experiments to inhibit PKC. Inhibition by the 'selective' PKC inhibitor Ro 31-8220 of Na^+ channel-dependent, but not of Na^+ channel-independent, glutamate release is consistent with the inhibition of presynaptic Na^+ channels. Thus, tetrodotoxin-sensitive glutamate release evoked by 4-aminopyridine or veratridine was inhibited by 10 μM Ro 31-8220 (by 50–70%), while tetrodotoxin-insensitive glutamate release evoked by elevated KCl or the Na^+/H^+ ionophore monensin was not inhibited. The mechanism of inhibition of 4AP- and veratridine-evoked glutamate release by Ro 31-8220 could involve: (1) blockade of presynaptic Na^+ channels required for terminal depolarization; (2) blockade of presynaptic Ca^{2+} channel subtype(s) coupled to glutamate release; (3) interference with synaptic vesicle fusion/release mechanisms; (4) inhibition of reversed Na^+/glutamate transport and/or (5) stimulation of glutamate reuptake. The absence of Ro 31-8220 effects on KCl- or monensin-evoked release suggests that inhibition of glutamate release involves primarily a blockade of Na^+ channels, though some contribution by the other mechanisms could also be involved. This interpretation is supported by the observations that Ro 31-8220, as well as the chemically related bisindolylmaleimides I and V [19], inhibited somatodendritic Na^+ currents measured by whole-cell patch-clamp recording of isolated DRG neurons. The effects of bisindolylmaleimides I,

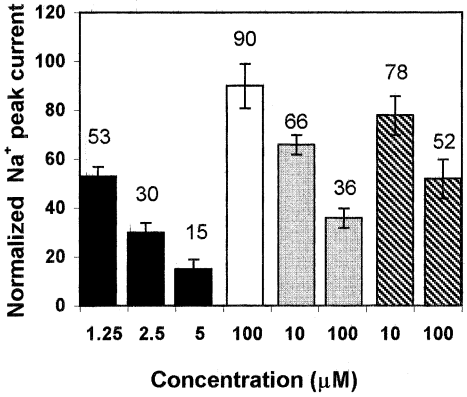

Fig. 3. Effects of various PKC inhibitors on whole-cell Na^+ current in isolated DRG neurons. Summary data are expressed as mean ± S.D. ($n = 3$) and normalized to control Na^+ current in the absence of drug. Black: Ro 31-8220; open: H7; gray: GF 1092037; hatched: bisindolylmaleimide V. All values, except those for H7, were significantly different from control ($P < 0.05$ by ANOVA).

IX (inhibitors of PKC) and V (inactive against PKC) suggest that a common structural feature of the bisindolylmaleimides distinct from that involved in PKC inhibition is involved in Na^+ channel inhibition.

Rat brain CNaIIa Na^+ channels expressed in CHO cells are themselves regulated by phosphorylation [20]. Several observations indicate that inhibition of Na^+ channels by bisindolylmaleimides is direct rather than a consequence of inhibition of channel phosphorylation by PKC: (1) phosphorylation by PKC inhibits channel activity [20]; (2) bisindolylmaleimide V (inactive), but not the structurally unrelated PKC inhibitor H7, inhibited Na^+ channels; (3) the onset of Na^+ inhibition by bisindolylmaleimides measured electrophysiologically was rapid (within 20 s of application) and (4) Ro 31-8220 inhibited [^{3}H]BTX-B binding. The latter two findings also make an effect mediated by inhibition of a Na^+ channel kinase other than PKC unlikely.

Inhibition of Na^+ channels must be added to the growing list of additional effects of these 'specific' PKC inhibitors, which now includes inhibition of other protein kinases [4,5], ligand-gated ion channels [6,7] and G protein-coupled receptors [8]. Ro 31-8220 and GF 109203X are potent inhibitors of the conventional isoforms of PKC with IC_{50} values in the nanomolar range [1,2]. Nevertheless, inhibition of PKC in intact cellular systems requires much higher inhibitor concentrations (i.e. micromolar) to overcome the effects of high intracellular PKC concentrations, high ATP concentrations and cell membrane permeability barriers. These higher inhibitor concentrations are able to inhibit a number of other targets in addition to PKC. This lack of specificity makes use of these inhibitors insufficient to implicate PKC in the regulation of a physiological process in the absence of corroborative data.

Despite these limitations, our results suggest an approach by which bisindolylmaleimide inhibitors can be used to probe the role of PKC in the regulation of physiological processes in excitable cells (which contain Na^+ channels). Inhibition of a process by a bisindolylmaleimide inhibitor (Ro 31-8220 or GF 109203X) alone is inconclusive. Inhibition of the process by both a bisindolylmaleimide inhibitor and by H7, but not by bisindolylmaleimide V, provides strong support that PKC is involved since H7 does not significantly inhibit Na^+ channels. Conversely, inhibition of the process by both a bisindolylmaleimide inhibitor and to a lesser extent by bisindolylmaleimide V, but not by H7, suggests a role for Na^+ channels rather than PKC in the process.

Studies that employ bisindolylmaleimide PKC inhibitors as specific pharmacological probes for PKC involvement must be reevaluated in light of these findings. For example, Coffey et al. [21] interpreted the observation that 10 μM Ro 31-8220 inhibited 4AP-evoked, but not elevated KCl-evoked, glutamate release from rat cortical synaptosomes as evidence that PKC plays a major role in regulating the depolarization of the terminal. Cousin et al. [22] interpreted the observation that 10 μM Ro 31-8220 inhibited electrical field-evoked, but not elevated KCl-evoked, [^{3}H]D-aspartate release from rat cerebellar granule cells as evidence that PKC plays a major role in field-evoked release. Our results suggest the alternative interpretation that these effects are due to Na^+ channel block by Ro 31-8220.

In conclusion, Ro 31-8220 and GF 109203X, which are often employed as specific inhibitors of PKC, are also able to directly block neuronal voltage-dependent Na^+ channels.

Acknowledgements: Supported by National Institutes of Health grant GM 58055, Bethesda, MD, USA (H.C.H.) and a fellowship from the Norman and Rosita Winston Foundation in Biomedical Sciences, New York, NY, USA (R.L.).

References

[1] Hemmings, H.C., Jr. (1997) in: Neuromethods (Hemmings, H.C., Jr., Ed.), Vol. 30, pp. 121–218. Humana, Totowa, NJ.
[2] Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E. and Loriolle, F. (1991) J. Biol. Chem. 266, 15771–15781.
[3] Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science 241, 42–52.
[4] Hers, I., Tavaré, J.M. and Denton, R.M. (1999) FEBS Lett. 460, 433–436.
[5] Alessi, D.R. (1997) FEBS Lett. 402, 121–123.
[6] Coultrap, S.J., Sun, H., Tenner, T.E. and Machu, T.K. (1999) J. Pharmacol. Exp. Ther. 290, 76–82.
[7] Marley, P.D. and Thomson, K.A. (1996) Br. J. Pharmacol. 119, 416–422.
[8] Lazareno, S., Popham, A. and Birdsall, N.J.M. (1998) Eur. J. Pharmacol. 360, 281–284.
[9] Dunkley, P.R., Jarrie, P.E., Heath, J.W., Kidd, G.J. and Rostas, J.A.P. (1986) Brain Res. 372, 115–129.
[10] Ratnakumari, L. and Hemmings Jr., H.C. (1997) Anesthesiology 86, 428–439.
[11] Nicholls, D.G., Talvinder, S.S. and Sanchez-Prieto, J. (1987) J. Neurochem. 49, 50–57.
[12] Nicholls, D.G. (1993) Eur. J. Biochem. 212, 613–631.
[13] Ratnakumari, L. and Hemmings Jr., H.C. (1996) Br. J. Pharmacol. 119, 1498–1504.
[14] Kostyuk, P.G., Veselovsky, N.S. and Tsyndrenko, A.Y. (1981) Neuroscience 6, 2423–2430.
[15] Scott, B.S. and Edwards, B.A. (1980) J. Neurobiol. 11, 291–301.
[16] Yoshida, S. (1994) Cell Mol. Neurobiol. 14, 227–244.
[17] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[18] Wilkinson, S.E., Parker, P.J. and Nixon, J.S. (1993) Biochem. J. 294, 335–337.
[19] Davis, P.D., Elliott, L.H., Harris, W., Hill, C.H., Hurst, S.A., Keech, E., Kumar, M.K., Lawton, G., Nixon, J.S. and Wilkinson, S.E. (1992) J. Med. Chem. 35, 994–1001.
[20] Li, M., West, J.W., Numann, R., Murphy, B.J., Scheuer, T. and Catterall, W.A. (1993) Science 61, 1439–1442.
[21] Coffey, E.T., Sihra, T.S. and Nicholls, D.G. (1993) J. Biol. Chem. 268, 21060–21065.
[22] Cousin, M.A., McLaughlin, M. and Nicholls, D.G. (1999) Eur. J. Neurosci. 11, 101–109.