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Paclitaxel and vinorelbine, evoked the release of substance P from cultured rat dorsal root ganglion cells through different PKC isoform-sensitive ion channels

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Abstract

Many patients suffer from serious adverse effects including respiratory distress and pulmonary edema during and after chemotherapy with paclitaxel or vinorelbine. These effects appear to be due to the activation of neurokinin-1 receptors. The present study investigated the influences of paclitaxel and vinorelbine on the substance P (sP) release from cultured dorsal root ganglion (DRG) cells using a radioimmunoassay. Both paclitaxel and vinorelbine evoked sP release in a dose- and time-dependent manner within 60 min at a concentration range of 0.1–10 μM. The sP release levels induced by the two drugs were attenuated by pretreatment with the protein kinase Cs (PKCs) inhibitors (bisindolylmaleimide I and Gö6976). Moreover, the paclitaxel- or vinorelbine-induced sP release was diminished in the absence of extracellular Ca^{2+} or the presence of $LaCl_3$ (an extracellular Ca^{2+} influx blocker). A Ca^{2+} imaging assay further indicated that both paclitaxel and vinorelbine gradually increased the intracellular Ca^{2+} concentration, and these increases lasted for at least 15 min and were suppressed by Gö6976. Paclitaxel caused the membrane translocation of only PKCβ within 10 min after stimulation, whereas vinorelbine induced the translocation of both PKCα and β. The paclitaxel- and vinorelbine-induced sP release levels were separately inhibited by ruthenium red (a transient receptor potential (TRP) channel blocker) and gabapentin (an inhibitor of voltage-gated Ca^{2+} channels (VGCCs)). These findings suggest that paclitaxel and vinorelbine evoke the sP release from cultured DRG cells by the extracellular Ca^{2+} influx through TRP channels activated by PKCβ and VGCCs activated by both PKCα and β, respectively.

Keywards: DRG cells; paclitaxel; PKC; sP; vinorelbine

Abbreviations: BIM, bisindolylmaleimide; cPKC, conventional protein kinase C; COX-2, cyclooxygenases-2; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; NK-1 receptor, neurokinin-1 receptor; nPKC, novel protein kinase C; PKC, protein kinase C; SDS, sodium dodecyl sulfate; sP, substance P; TRP channel, transient receptor potential channel; TRPV, transient receptor potential channel vanilloid; VGCCs, voltage-gated Ca^{2+} channels;

1. Introduction

Both paclitaxel and vinorelbine are the most effective and commonly used anti-cancer drugs for breast cancer and non-small cell lung carcinoma. Following binding to β-tubulin, both paclitaxel and vinorelbine separately interfere with dynamic assembly or disassembly of the mitotic spindle, and thereby arrest the cell division process (Jordan and Wilson, 2004). During chemotherapy, however, the two drugs frequently cause severe hypersensitive reactions, such as bronchospasm, pulmonary edema and respiratory distress (Weiss et al., 1990). Initially, the induction of these undesirable adverse effects was considered to be associated with the histamine release from mast cells (Decorti et al., 1996). However, premedication using a combination of histamine H_1 and H_2 antagonists with glucocorticoids is not always able to prevent these hypersensitive responses triggered by paclitaxel (Bookman et al, 1997). Therefore, it is suggested that other mediator(s) must induce these hypersensitive responses.

Substance P (sP) is a member of the tachykinin neuropeptide family, which is released from the central and peripheral nerve terminals of primary sensory neurons in response to noxious stimuli (Severini et al., 2002; Tang et al., 2006). It is well known that sP binds with high affinity to a receptor called neurokinin-1 (NK-1) receptor. At the spinal terminals of primary neurons, sP plays a role in the transmission of nociceptive information to the central nervous system, while the sP released at the peripheral region functions as a neuromodulator and induces vasodilation, the constriction of smooth muscle, submucosal gland secretion and inflammation (Severini et al., 2002). Therefore, sP seems to be a major initiator of neurogenic inflammation and contributes to various diseases (O'Connor et al., 2004). For example, Sendo et al. (2004) reported an elevated plasma level of sP in patients at 10 min after paclitaxel infusion. In addition, the injection of paclitaxel increases the sP level in plasma and bronchoalveolar lavage fluid, and caused not only a marked extravasation of plasma protein in the lungs but also a

decrease in arterial partial oxygen pressure with a concomitant increase in arterial partial carbonic anhydride (Itoh et al., 2004a and b; Sendo et al., 2004). Moreover, both the plasma extravasation in the lungs and the respiratory dysfunction induced by paclitaxel are reversed by an antagonist of NK-1 receptor (Itoh et al., 2004a and b). These observations imply that sP might play an important role in the clinical hypersensitive responses including pulmonary edema and respiratory distress during the infusion of paclitaxel or vinorelbine. However, there is still no direct evidence that both paclitaxel and vinorelbine influence the sP release from the primary afferent neurons. The present study was therefore designed to investigate whether, and if so, how both paclitaxel and vinorelbine induce the sP release from cultured dorsal root ganglion (DRG) cells.

2. Materials and Methods

2.1. Materials

The following drugs were used: bisindolylmaleimide I, Gö6976, PKCε translocation inhibitor peptide and paclitaxel (Calbiochem, La Jolla, CA, USA); lanthanum chloride ($LaCl_3 \cdot 7H_2O$) (Nakarai Tesque, Kyoto, Japan); Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan); horse serum and glutamine (Gibco-BRL, Gaithersburg, MD, USA); 2.5% trypsin (Invitrogen, Burlington, ON, Canada); amiloride, bacitracin, bovine serum albumin, capsazepine, captopril, collagenase, gabapentin, Hanks' balanced salt solution, nicardipine, penicillin/streptomycin, phosphoramidon, polyethyleneimine and ruthenium red (Sigma Chemical, St Louis, MO, USA); nerve growth factor (2.5 S) (Promega, Madison, WI, USA); fura-2 acetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan); [^{125}I-Try^{8}]-sP (81.4 TBq/mmol) (New England Nuclear, Boston, MA, USA); cell compartment kit (Qiagen, Hilden, Germany); mouse antibodies IgG against PKCα and PKCβ, and mouse laminin (BD biosciences, Bedford, MA, USA);

anti-mouse IgG horseradish peroxidase-linked antibody (Cell signaling, Beverly, MA, USA); ω-conotoxin GVIA (Peptide Institute, Osaka, Japan). Anti-cancer drugs including dacarbazine (a non-classical alkylating agent), fluorouracil (a pyrimidine analog), mitomycin C (an antibiotic) and vinorelbine were kindly provided by Kyowa Hakko (Tokyo, Japan). All other reagents were of the highest purity available from commercial sources.

2.2. Cell culture

According to a previously described method (Tang et al., 2006), DRGs were obtained from male Wistar rats weighing from 200–300 g and dissociated into single cells by enzyme treatments of 0.125% collagenase and 0.25% trypsin, and mechanical trituration. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 4 mM glutamine, 1% penicillin/streptomycin, and 30 ng/ml nerve growth factor, and plated on polyethyleneimine and laminin-coated dishes at density of 5–6 DRGs/35 mm dish. The cultures were maintained at 37^{o}C in a water-saturated atmosphere with 5% CO_2 for 3–7 days before the initiation of experiments. All animal procedures were performed in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University, Japan.

2.3. Measurement of sP release

All experiments were performed in Krebs–HEPES buffer [NaCl 110, $CaCl_2$ 2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.7, HEPES 5 (mM)] contained 0.1% bovine serum albumin and peptidase inhibitors (1 μM phosphoramidon, 1 μM captopril and 4 μg/ml bacitracin). Ca^{2+}-free Krebs–HEPES buffer was prepared by

omitting $CaCl_2$. After washing, the cultured DRG cells were pretreated with various inhibitors (amiloride, bisindolylmaleimide I, $LaCl_3$ and capsazepine, ω-conotoxin GVIA, gabapentin and Gö6976, nicardipine, PKCε translocation inhibitor peptide and ruthenium red) for 15 min at 37°C. Thereafter, these cells were continuously treated with anti-cancer drugs (paclitaxel, vinorelbine, dacarbazine, fluorouracil and mitomycin C) for the designated period of time (10, 30 and 60 min) at 37°C. The content of sP collected from Krebs–HEPES buffer was measured by a sensitive radioimmunoassay (Tang et al., 2006).

2.4. Cell fractionation and Western blot analysis

After the treatment of cultured DRG cells with 10 μM of either paclitaxel or vinorelbine for the designated period of time (0, 2, 5 and 10 min) in Krebs–HEPES buffer, the isolation of the membrane and cytosolic protein fractions from those cells was performed according to the manufacturer's instructions of the cell compartment kit (Qiagen, Hilden, Germany). Thereafter, the protein samples were diluted in sodium dodecyl sulfate (SDS) sample buffer, and sonicated. After heating at 95°C for 5 min, equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. The membranes were blocked with blocking buffer containing 5% skim milk overnight at 4°C and incubated for 2 hr at room temperature with primary mouse IgG antibodies against PKCα (1:1000) and PKCβ (1:250), respectively. After washing, the membranes were further incubated with horseradish peroxidase-linked anti-mouse IgG antibody (1:1000) for 1 hr at room temperature. The immunoreactivity was detected using the ECL plus Western blotting detection system (GE Healthcare, Little Chalfont, Bucks, UK). Finally, the band densities of PKC isoforms in membrane and cytosol fractions were measured using a computerized image analysis program (Scion Image Beta 4.0.3; Scion Corporation,

Frederick, MD, USA). The extent of membrane translocation of the PKC isoforms was calculated using the ratio of the PKC isoform-specific band density (membrane/cytosol), according to a previously described method (Pan et al., 2008).

2.5. Measurement of intracellular Ca^{2+} concentration

All experiments were performed in Hanks' balanced salt solution (Sigma Chemical, St Louis, MO, USA). The cultured DRG cells plated on glass coverslips with a silicon rubber wall (FlexiPERM; Heraeus Biotechnology, Hanau, Germany) were loaded with 10 μM of fura-2 acetoxymethyl ester for 60 min at room temperature. The fluorescence intensity was measured with the excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The video image output was digitized by an Argus 50 color image processor (Hamamatsu Photonics, Shizuoka, Japan) as described elsewhere (Hide et al., 1997).

2.6. Statistical analysis

The data are presented as the mean ± SEM of at least three independent experiments. The statistical analysis of the data was performed by a one-way analysis of variance (ANOVA) followed by Dunnett's test. A probability value (p) of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Both paclitaxel and vinorelbine induced the sP release from cultured rat DRG cells

As shown in Fig.1A, treatment with 10 μM of either paclitaxel or vinorelbine induced a time-dependent release of sP within 60 min after stimulation. Treatment with either paclitaxel or vinorelbine at a concentration range of 0.1–10 μM for 30 min induced the sP release in a dose-dependent manner (Fig.1B). In contrast, other kinds of

anti-cancer drugs including dacarbazine, fluorouracil and mitomycin C did not affect the release of sP (Fig. 1B). Therefore, the cultured DRG cells treated with 10 μM of either paclitaxel or vinorelbine for 30 min were used to further investigate the mechanism of sP release from cultured DRG cells.

3.2. Involvement of protein kinase C activation on the paclitaxel- and vinorelbine-induced sP release

Previous studies have demonstrated the importance of various intracellular cell signaling factors including protein kinase C (PKC) in the regulation of sP release (Tang et al., 2007 and 2008). Therefore, the effects of PKC inhibitors on the sP release induced by either paclitaxel or vinorelbine were examined. As shown in Fig. 2, both the paclitaxel- and vinorelbine-induced sP release were significantly attenuated by the pretreatment with bisindolylmaleimide I, an inhibitor of conventional PKC (cPKC) and novel PKC (nPKC) (Martiny-Baron et al., 1993), or Gö6976, an inhibitor of cPKC (Martiny-Baron et al., 1993), but not by PKCε translocation inhibitor peptide (Huang and Walker, 2004). The preincubation with these inhibitors alone had no significant effect on the sP release.

The cPKCs are divided into three isoforms: PKCα, β and γ (Liu and Heckman, 1998). In a separate study, the expression of PKCα and β, but not PKCγ has been confirmed in cultured DRG cells (Unpublished data). Therefore, the effect of both paclitaxel and vinorelbine on the translocation of PKCα and β from cytosol to membrane, which is one of the hallmarks of cPKC activation (Liu and Heckman, 1998), were investigated. Vinorelbine, but not paclitaxel increased the level of PKCα expression in the membrane fraction which peaked at the 2-min time point (Figs. 3A and B). This translocation from the cytosol to membrane returned toward the basal level at the 10-min time point. On the other hand, the level of PKCβ in the membrane fraction was increased by the

treatment with paclitaxel or vinorelbine (Figs. 3C and D). The translocation of PKCβ continued for at least 10 min after the stimulation with either paclitaxel or vinorelbine (Figs. 3C and D).

3.3. Influx of extracellular Ca^{2+} through ion channels is essential for the sP release induced by either paclitaxel or vinorelbine

The sP release may be modulated by the influx of extracellular Ca^{2+} through several ion channels, such as transient receptor potential (TRP) channels and voltage-gated Ca^{2+} channels (VGCCs) (Kageyama et al., 1997; Oshita et al., 2005; Tang et al, 2006; Tang and Nakata, 2008; Trevisani et al., 2007). Therefore, the involvement of extracellular Ca^{2+} was investigated in both the paclitaxel- and vinorelbine-induced sP release. As shown in Fig. 4, neither paclitaxel nor vinorelbine affected the sP release in the absence of extracellular Ca^{2+}. In addition, preincubation with $LaCl_3$ (300 μM), which blocks Ca^{2+} influx (Robertson et al., 1995), completely suppressed the sP release induced by either paclitaxel or vinorelbine. Next, the effects of several inhibitors of TRP channels and VGCCs on the paclitaxel- and vinorelbine-induced sP release were examined, because the sensitization of these channels may be modulated by PKC including cPKC (Dolphin, 1995; Fehrenbacher et al., 2003; Venkatachalam and Montell, 2007; Tang et al., 2008). As shown in Figs. 5A and B, preincubation with 50 μM ruthenium red, a non-selective inhibitor of TRP channels (Patapoutian et al., 2003; Qin et al., 2008), significantly suppressed the sP release induced by paclitaxel, but not vinorelbine. However, pretreatment with 1 μM capsazepine, an inhibitor of TRP channel vanilloid subtype 1 (TRPV1) (Bevan et al., 1992), had no significant effect on both paclitaxel- and vinorelbine-induced sP release. On the other hand, the pretreatment with 50 μM gabapentin, a non-selective inhibitor of VGCCs (Gee et al., 1996; Martin et al., 2002), significantly attenuated the sP release induced by vinorelbine, but not paclitaxel (Figs.

5C and D). However, the pretreatment with the respective inhibitors of L-, N- and T-type VGCCs, such as nicardipine, ω-conotoxin GVIA and amiloride (Waterson, 1996; Hui et al., 2009; Shishido et al., 2009), was not able to significantly prevent either the paclitaxel- or vinorelbine-induced sP release (Figs. 5C and D).

3.4. Increases in the concentration of intracellular Ca^{2+} induced by paclitaxel or vinorelbine

The effects of paclitaxel and vinorelbine on the intracellular Ca^{2+} concentration were examined in cultured DRG cells. As shown in Figs. 6A and B, both paclitaxel and vinorelbine increased the intracellular Ca^{2+} concentration. The increase in the intracellular Ca^{2+} concentration induced by paclitaxel took about 5 min after the stimulation, whereas the vinorelbine-induced increase started at 2 min after the stimulation. Therefore, the effects of various inhibitors which affected sP release in this study on the increase in the intracellular Ca^{2+} concentration induced by either paclitaxel or vinorelbine were investigated. The increase in the intracellular Ca^{2+} concentration induced by paclitaxel or vinorelbine was completely abolished by the elimination of extracellular Ca^{2+} (Figs. 6C and D). Pretreatment with $LaCl_3$ (Figs. 6E and F) or Gö6976 (Figs. 6G and H) also inhibited the increase in the intracellular Ca^{2+} concentration induced by either paclitaxel or vinorelbine. In addition, the paclitaxel-induced increase in the intracellular Ca^{2+} concentration was suppressed by the pretreatment with ruthenium red, but not gabapentin (Figs. 6I and K). On the other hand, the pretreatment with gabapentin, but not ruthenium red, attenuated the vinorelbine-induced increase in the intracellular Ca^{2+} concentration (Figs. 6J and L).

4. Discussion

This study demonstrated for the first time that both paclitaxel and vinorelbine

induced the sP release from cultured rat DRG cells in a time- and dose-dependent manner by the influx of extracellular Ca^{2+} through the distinct ion channels activated by different isoforms of cPKCs. In addition, other kinds of anti-cancer drugs (dacarbazine, fluorouracil, and mitomycin C), which do not particularly cause hypersensitive reactions during cancer chemotherapy, did not significantly influence the sP release (Fig. 1B). Therefore, these data suggest that the sP release seems to be considered as a specific indicator of the hypersensitive reactions triggered by anti-mitotic drugs, paclitaxel and vinorelbine.

Frayer et al. (1999) have indicated that a PKC activator, phorbol 12, 13-dibtyrate, evoked the sP release in spinal cord slices, thus suggesting that the activation of PKC is very important in sP release. The present study demonstrated that the effect of paclitaxel as well as vinorelbine on the sP release was dependent on the activation of PKC (Fig. 2). The current study used 1 μM Gö6976 for the inhibition of the activity of cPKC, because 1 μM Gö6976 has no effect on the activity of nPKC and atypical PKC (Martiny-Baron et al., 1993). Therefore, the concentration of Gö6976 used in this study is appropriate to selectively inhibit the activity of cPKCs. PKCε, a member of the nPKCs, plays a major role in the sP release from cultured DRG cells (Tang and Nakata, 2008; Tang et al., 2008), however, the pharmacological blockade of PKCε did not inhibit either the paclitaxel- or vinorelbine-induced sP release (Fig. 2). Taken together, these data suggest that the mechanisms of both the paclitaxel- and vinorelbine-induced sP release might be regulated by the activation of cPKCs rather than nPKCs.

Subbaramaiah et al. (2000) showed the paclitaxel-induced expression of cyclooxygenase-2 (COX-2) protein and mRNA in human mammary epithelial cells to be blocked by calphostin C, an inhibitor of PKC, thus suggesting that the activation of PKC might be activated by paclitaxel. The present study indicated that paclitaxel activated only PKCβ, whereas vinorelbine activated both PKCα and β (Fig. 3), thus the

cPKC isoforms activated by paclitaxel and vinorelbine were distinct in cultured DRG cells. The activation of PKCα induced by vinorelbine rapidly peaked and thereafter returned to the basal level. The time course and the extent of PKCβ activation induced by paclitaxel seem similar to vinorelbine (Figs. 3C and D). These data suggest: 1) the site of action of paclitaxel on cultured DRG cells might be different from vinorelbine in terms of the sP release; 2) vinorelbine is likely to have an additional action site which is not affected by paclitaxel. Moreover, vinorelbine seems to have augmentative action on the sP release through the activation of PKCα in comparison to paclitaxel, since the amount of the vinorelbine-induced sP release was larger than that of paclitaxel (Fig. 1A). Taken together, these data suggest that the paclitaxel-induced sP release might be regulated by the activation of PKCβ, whereas vinorelbine could induced the sP release through the activation of both PKCα and β.

Intracellular Ca^{2+} is an important second messenger in regulating various cell responses including the sP release (Oshita et al. 2005; Tang et al., 2006; Tang and Nakata, 2008). In the present study, both paclitaxel and vinorelbine gradually induced the increase in intracellular Ca^{2+} concentration. These increases took several minutes to peak after stimulation with paclitaxel or vinorelbine (Figs. 6A and B). Moreover, pretreatment with Gö6976 suppressed the increase in the intracellular Ca^{2+} concentration induced by paclitaxel or vinorelbine (Figs. 6G and H), and the time courses of the activation of cPKCs stimulated by the two drugs were rapid, within 10 min after stimulation (Fig. 3). These data suggest that paclitaxel and vinorelbine could activate cPKCs first, and then induce the influx of extracellular Ca^{2+}, and therefore the influx of extracellular Ca^{2+} appears to be essential for both the paclitaxel- and vinorelbine-induced sP release.

This study found that the involvement of the TRP channel family in the paclitaxel-release, but not the vinorelbine-induced sP release, because ruthenium red,

which is commonly used as a non-selective inhibitor of TRP channels (Patapoutian et al., 2003), has substantial inhibitory effects on the paclitaxel-induced actions (Figs. 5 and 6). Previous studies have demonstrated that the activation of TRPV1 closely contributes to the sP release from cultured DRG cells (Tang et al., 2006 and 2008; Tang and Nakata, 2008). Therefore, TRPV1 could be one of the candidate TRP channels associated with the paclitaxel-induced actions. However, this hypothesis may be excluded, because capsazepine had no significant inhibitory effect on the paclitaxel-induced release (Fig. 5A). Other studies have indicated that DRG neurons express the other TRP channels including TRPA1, TRPV3, 4 and TRPM8, thus suggesting that the activation of these TRP channels may be involved in the sP release from DRG neurons (Facer et al., 2007; Grant et al., 2007; Trevisani et al., 2007). Taken together, these data suggest that the paclitaxel-induced sP release might be evoked by the influx of extracellular Ca^{2+} through TRP channels other than TRPV1.

VGCCs are composed by heteromultimeric proteins containing a pore forming α1 subunit and auxiliary β, α2/δ and γ subunits, and divided into six groups (L-, N- P-, Q-, R-, and T-types) based on the properties of the α1 subunit (Dolphin, 1995). A previous study showed that sP and several types of VGCCs are co-expressed in the DRG cells (Scroggs and Fox, 1992). In practice, we also found that the pretreatment with gabapentin, a selective and potent inhibitor of VGCCs (Gee et al., 1996; Martin et al., 2002), caused a significant inhibition of the sP release and the intracellular Ca^{2+} concentration induced by vinorelbine, but not paclitaxel (Figs. 5 and 6). However, we considered that the L-, N- and T-type VGCCs may not be involved in the vinorelbine-induced sP release because none of the three VGCC inhibitors (nicardipine, ω-conotoxin and amiloride) significantly attenuated the vinorelbine-induced sP release (Fig. 5D). Therefore, it is suggested that several VGCCs other than the L-, N- and T-types regulate the vinorelbine-induced Ca^{2+} influx, thus triggering the sP release from

cultured DRG cells.

The present study does not account for the relationship between PKC and ion channels (TRP channels and VGCCs). However, VGCCs possess the phosphorylation sites for PKC, and the activition of VGCCs may be modulated by PKCs (Dolphin, 1995). Recent reports have also suggested that the sensitization of TRP channels other than TRPV1 was also regulated by the activation of PKC (Grant et al., 2007; Venkatachalam and Montell, 2007). Moreover, Kashihara et al. (2008) indicated that the PKC isoforms play a distinct role in the extracellular Ca^{2+} influx through TRP channels or VGCCs. Our present study therefore suggested that paclitaxel might open the TRP channels through the activation of PKCβ, while vinorelbine could activate VGCCs through both PKCα and β, thus indicating that this distinct mechanism may be due to the difference in the cPKCs activation induced by the two drugs and the transient PKCα activation induced by vinorelbine is likely to be important key for elucidating the distinct mechanism of these two drugs. However, further investigations are still needed to determine the isoforms of TRP channels and VGCCs and the distinct molecules in these channels which contribute to the modulation of the paclitaxel- and vinorelbine-induced effects.

In conclusion, both paclitaxel and vinorelbine may cause an extracellular Ca^{2+} influx through the TRP channels or VGCCs dependent on the activation of respective cPKC isoforms (PKCβ and/or α), thus leading to an increase in the intracellular Ca^{2+} concentration, and thereby evoke the sP release from cultured DRG cells. These observations provide an important key to substantially reduce or even prevent the paclitaxel- and vinorelbine-triggered side effects during and after chemotherapy.

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Legends

Fig. 1 The effects of various anti-cancer drugs on the sP release from cultured rat DRG cells.

(A) The time courses of the paclitaxel- and vinorelbine-induced sP release. The cells were treated with 10 μM of either paclitaxel or vinorelbine for the indicated periods of time. (B) The concentration dependency of the sP release induced by various anti-cancer drugs. The cells were treated with the indicated concentration of paclitaxel, vinorelbine, dacarbazine, fluorouracil and mitomycin C for 30 min. The data are expressed as the means ± S.E.M. (*bars*) of 4–14 (A) or 3–21 (B) separate experiments.*$p < 0.05$, **$p < 0.01$ in comparison to respective control groups.

Fig. 2 Effects of PKC inhibitors on the paclitaxel- or vinorelbine-induced sP release from cultured rat DRG cells.

The cells were pretreated with 10 μM of bisindolylmaleimide I (BIM), 1 μM of Gö6976 or 200 μM of PKCε translocation inhibitor peptide (PKCε I) for 15 min. After the pretreatment, the cells were treated with 10 μM of either paclitaxel or vinorelbine for 30 min, respectively. The data are expressed as the means ± S.E.M. (*bars*) of 3–10 (A) or 3–12 (B) separate experiments. *, ** denote $p < 0.05$ and 0.01 in comparison to the value for the cells treated with paclitaxel or vinorelbine alone.

Fig. 3 The translocation of cPKC isoforms from the cytosol to the membrane in cultured DRG cells treated with paclitaxel or vinorelbine.

The cells were treated with 10 μM of either paclitaxel (A, B) or vinorelbine (C, D) for the indicated period of time (0, 2, 5 and 10 min). The cPKC isoforms in the membrane and cytosol fractions were detected by a Western blot analysis (upsides of A, B, C and D), and the extent of the translocation of PKCα (downsides of C and D) or β

(downsides of C and D) was normalized to the control group treated with paclitaxel or vinorelbine for 0 min to 100%. The data are expressed as the means ± S.E.M. (*bars*) of 5–8 (A), 5–8 (B), 4 (C) or 6 (D) separate experiments. *$p < 0.05$, *$p < 0.01$ in comparison to the value for the cells treated with paclitaxel or vinorelbine at 0 min.

Fig. 4 Effects of extracellular Ca^{2+} on the paclitaxel- or vinorelbine-induced sP release.

The cultured DRG cells were treated with 10 μM of either paclitaxel or vinorelbine in the presence or absence of Ca^{2+} (2 mM) in Krebs–HEPES buffer. The experiments of $LaCl_3$ were preformed in Ca^{2+} (2 mM)-containing buffer. The data are expressed as the means ± S.E.M. (*bars*) of 3–6 separate experiments. **$p < 0.01$ in comparison to the value of paclitaxel or vinorelbine alone in the Ca^{2+}-containing buffer.

Fig. 5 Effects of inhibitors of ion channels on the paclitaxel- or vinorelbine-induced sP release from cultured rat DRG cells.

The cells were pretreated with 50 μM of ruthenium red (RR), 1 μM of capsazepine (CPZ) or ω-conotoxin GVIA (GVIA), 50 μM of gabapentin (GBP), 5 μM of nicardipine (Nic) or 100 μM of amiloride (AMD) for 15 min. After the preincubation, the cells were treated with 10 μM of either paclitaxel or vinorelbine for 30 min, respectively. The data are expressed as the means ± S.E.M. (*bars*) of 4–11 (A), 4–10 (B), 3–21 (C) or 3–23 (D) separate experiments. *, ** denote $p < 0.05$ and 0.01 in comparison to the value of the cells treated with paclitaxel or vinorelbine alone.

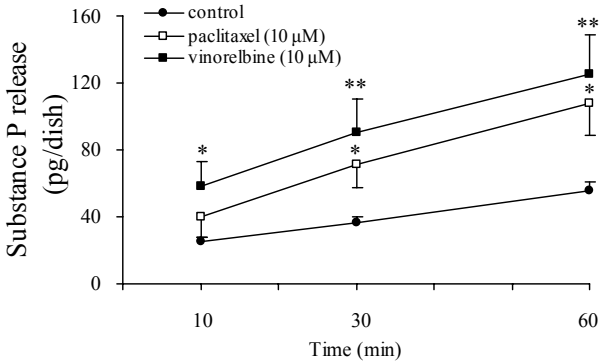
Fig. 6 The changes of the intracellular Ca^{2+} concentration in cultured DRG cells treated with paclitaxel or vinorelbine.

The fura-2-loaded cells were treated with 10 μM of either paclitaxel or vinorelbine in the presence (A, B, E-L) or absence (C, D) of Ca^{2+} in Hanks' balanced salt solution,

respectively. The cells were pretreated with 300 μM of $LaCl_3$ (E, F), 1 μM of Gö6976 (G, H), 50 μM of ruthenium red (I, J) or 50 μM of gabapentin (K, L) for 15 min, before the treatment with 10 μM of either paclitaxel or vinorelbine for 15 min. The trace in each graph shows the representative mean of the intracellular Ca^{2+} concentration of randomly selected cells. Similar results were obtained in at least three independent experiments.

Fig. 1

A


control
paclitaxel (10 μM)
vinorelbine (10 μM)
Substance P release (pg/dish)
160
120
80
40
0
10
30
60
Time (min)
*
**

B

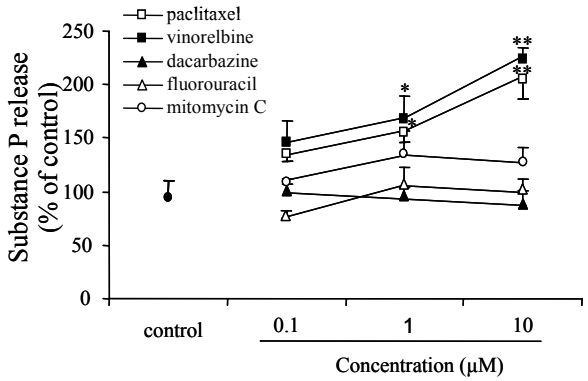
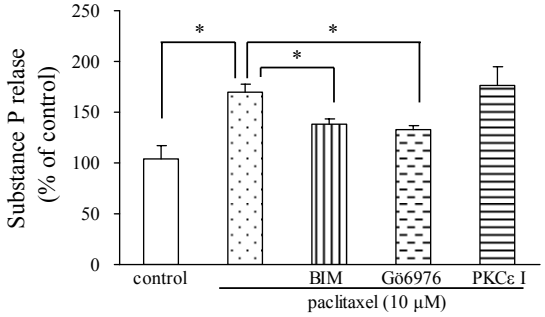

paclitaxel
vinorelbine
dacarbazine
fluorouracil
mitomycin C
Substance P release (% of control)
250
200
150
100
50
0
control
0.1
1
10
Concentration (μM)
*
**

Fig. 2

A


Substance P relase
(% of control)
250
200
150
100
50
0
*
*
*
control
BIM
Gö6976
PKCε I
paclitaxel (10 μM)

B

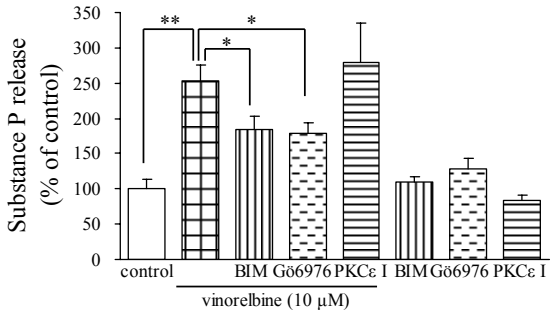
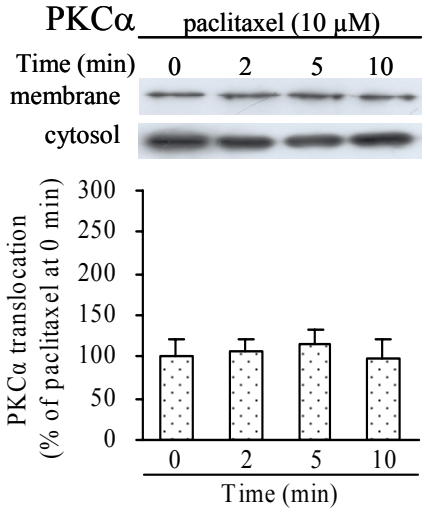
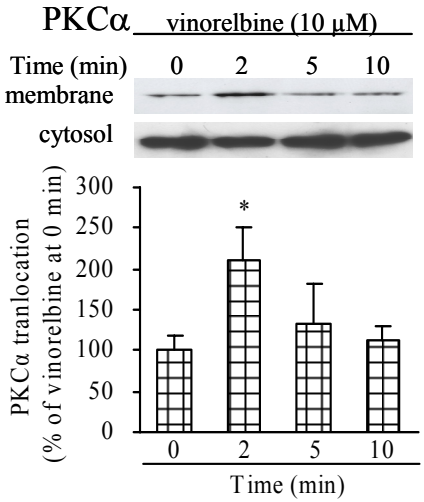

Substance P release
(% of control)
350
300
250
200
150
100
50
0
**
*
*
control
BIM
Gö6976
PKCε I
BIM
Gö6976
PKCε I
vinorelbine (10 μM)

Fig. 3

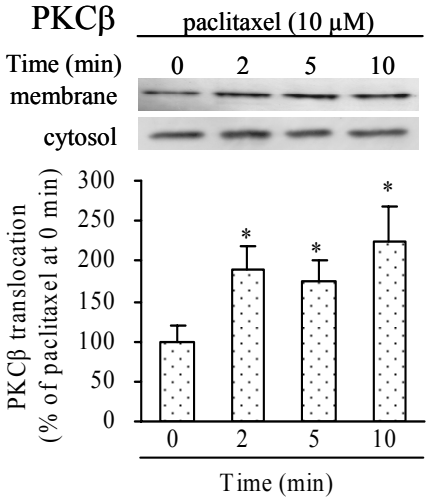
A


PKCα
paclitaxel (10 μM)
Time (min) 0 2 5 10
membrane
cytosol
PKCα translocation (% of paclitaxel at 0 min)
300
250
200
150
100
50
0
0 2 5 10
Time (min)

B


PKCα
vinorelbine (10 μM)
Time (min) 0 2 5 10
membrane
cytosol
PKCα tranlocation (% of vinorelbine at 0 min)
300
250
200
150
100
50
0
*
0 2 5 10
Time (min)

C


PKCβ
paclitaxel (10 μM)
Time (min) 0 2 5 10
membrane
cytosol
PKCβ translocation (% of paclitaxel at 0 min)
300
250
200
150
100
50
0
* * *
0 2 5 10
Time (min)

D

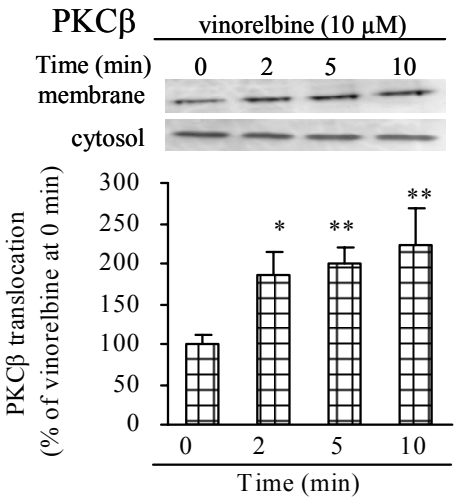
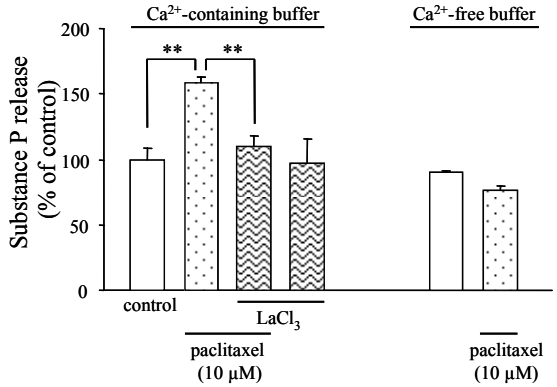

PKCβ
vinorelbine (10 μM)
Time (min) 0 2 5 10
membrane
cytosol
PKCβ translocation (% of vinorelbine at 0 min)
300
250
200
150
100
50
0
* ** **
0 2 5 10
Time (min)

Fig. 4

A


Ca2+-containing buffer
Ca2+-free buffer
200
150
100
50
0
**
**
Substance P release
(% of control)
control
LaCl3
paclitaxel
(10 μM)
paclitaxel
(10 μM)

B

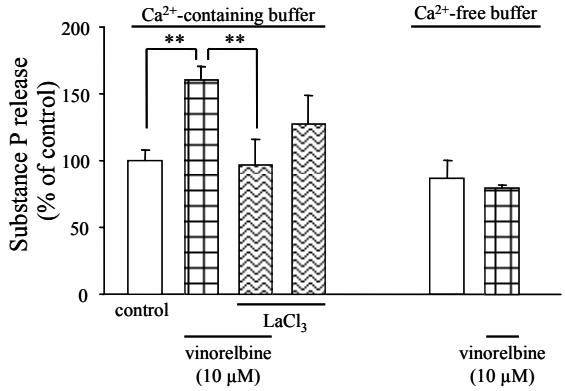
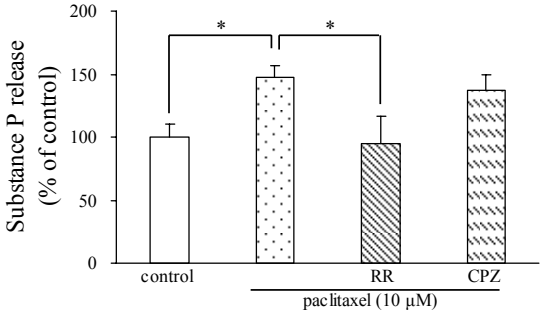
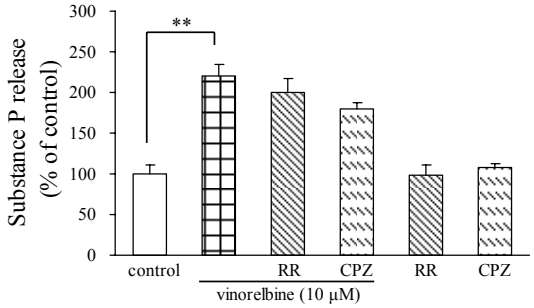

Ca2+-containing buffer
Ca2+-free buffer
200
150
100
50
0
**
**
Substance P release
(% of control)
control
LaCl3
vinorelbine
(10 μM)
vinorelbine
(10 μM)

Fig. 5

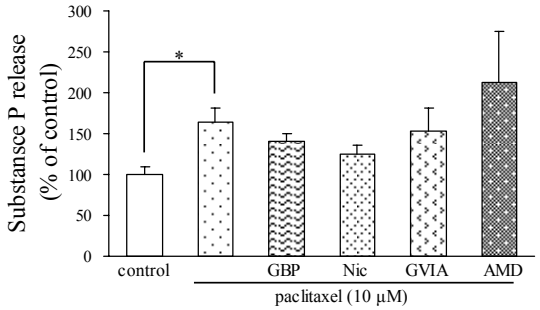
A


Substance P release
(% of control)
200
150
100
50
0
*
*
control
RR
CPZ
paclitaxel (10 μM)

B


Substance P release
(% of control)
300
250
200
150
100
50
0
**
control
RR
CPZ
RR
CPZ
vinorelbine (10 μM)

C


Substansce P release
(% of control)
300
250
200
150
100
50
0
*
control
GBP
Nic
GVIA
AMD
paclitaxel (10 μM)

D

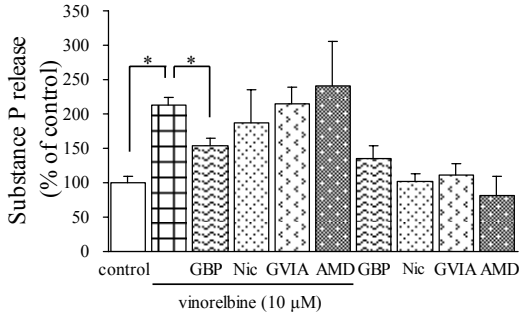
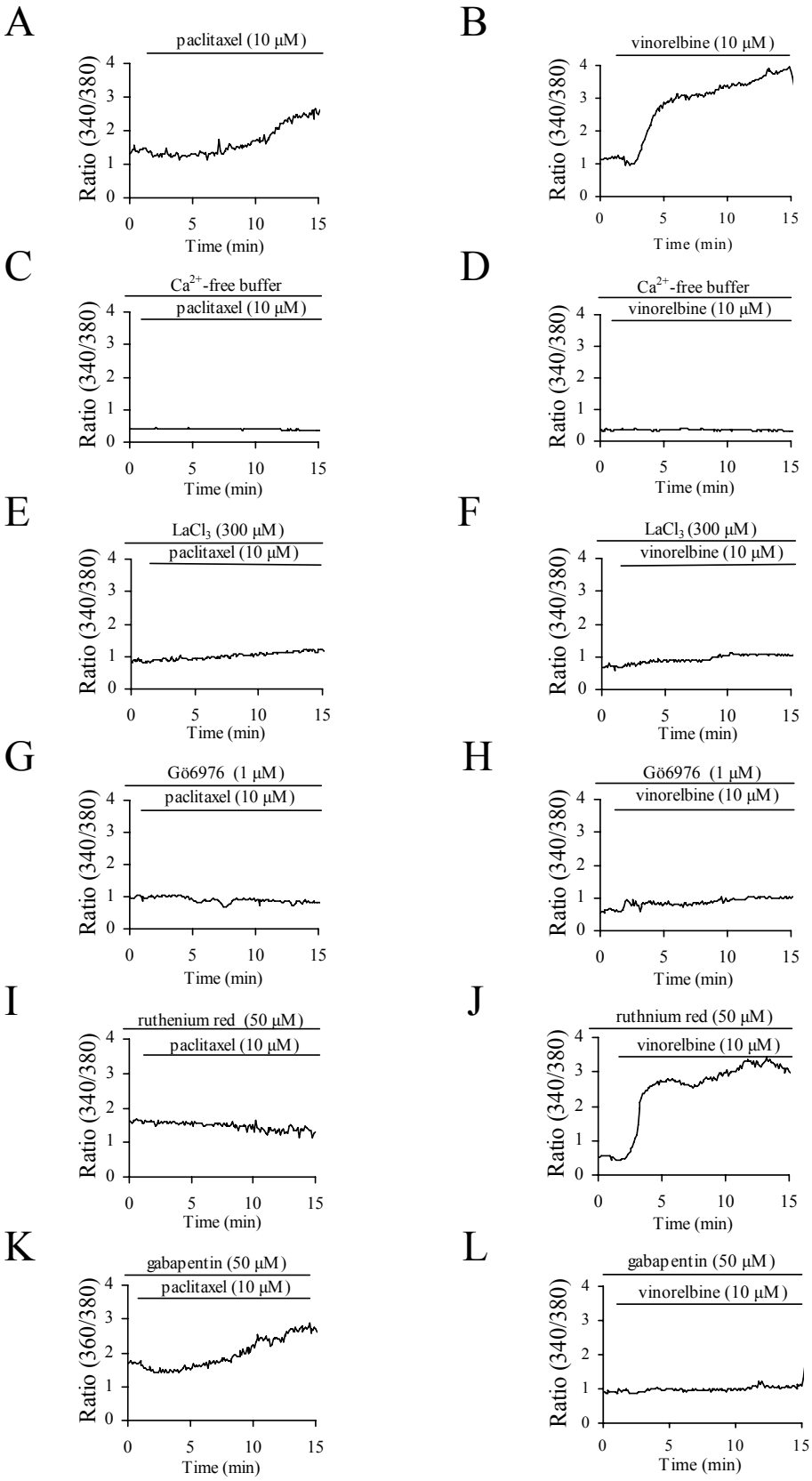

Substance P release
(% of control)
350
300
250
200
150
100
50
0
*
*
control
GBP
Nic
GVIA
AMD
GBP
Nic
GVIA
AMD
vinorelbine (10 μM)

Fig. 6


A
paclitaxel (10 μM)
Ratio (340/380)
Time (min)
B
vinorelbine (10 μM)
Ratio (340/380)
Time (min)
C
Ca2+-free buffer
paclitaxel (10 μM)
Ratio (340/380)
Time (min)
D
Ca2+-free buffer
vinorelbine (10 μM)
Ratio (340/380)
Time (min)
E
LaCl3 (300 μM)
paclitaxel (10 μM)
Ratio (340/380)
Time (min)
F
LaCl3 (300 μM)
vinorelbine (10 μM)
Ratio (340/380)
Time (min)
G
Gö6976 (1 μM)
paclitaxel (10 μM)
Ratio (340/380)
Time (min)
H
Gö6976 (1 μM)
vinorelbine (10 μM)
Ratio (340/380)
Time (min)
I
ruthenium red (50 μM)
paclitaxel (10 μM)
Ratio (340/380)
Time (min)
J
ruthnium red (50 μM)
vinorelbine (10 μM)
Ratio (340/380)
Time (min)
K
gabapentin (50 μM)
paclitaxel (10 μM)
Ratio (360/380)
Time (min)
L
gabapentin (50 μM)
vinorelbine (10 μM)
Ratio (340/380)
Time (min)