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Desensitization and resensitization of δ-opioid receptor-mediated Ca^{2+} channel inhibition in NG108-15 cells

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1 To approach the mechanisms underlying desensitization of the opioid receptor-mediated Ca^{2+} channel inhibition, the effects of prolonged application of [D-Ala^2, D-Leu^5]enkephalin (DADLE) on Ba^{2+} currents (I_{Ba}) through Ca^{2+} channels were analysed in NG108-15 neuroblastoma × glioma hybrid cells.

2 Inhibition of I_{Ba} by 100 nM DADLE desensitized by 57% with a time constant of 4.4 min.

3 Maximal desensitization of the δ-opioid receptor-Ca^{2+} channel coupling was attained by 1 μM DADLE. The EC_{50} value for desensitization was estimated to be 78 nM.

4 RNA blot hybridization analysis and immunoblot analysis revealed the expression of β-adrenoceptor kinase-1 (βARK1) in NG108-15 cells.

5 Heparin, an inhibitor of βARK, significantly reduced the magnitude and rate of desensitization, whereas Rp-cyclic AMPS and PKI (14-24)amide, inhibitors of cyclic AMP-dependent protein kinase (PKA), or long-term treatment with phorbol 12-myristate 13-acetate to induce down-regulation of protein kinase C (PKC) had no significant effect.

6 Recovery from desensitization (resensitization) proceeded with a time constant of 6.7 min. Okadaic acid, an inhibitor of serine/threonine phosphatases 1 and 2A, significantly attenuated the degree of resensitization.

7 In summary, we have characterized the time course and concentration-dependence of the desensitization of DADLE-induced I_{Ba} inhibition in NG108-15 cells. This desensitization was reversible after removal of DADLE. It is suggested that βARK, but neither PKA nor PKC, is involved in desensitization, while serine/threonine phosphatases mediate resensitization.

Keywords: Opioids; opioid δ receptors; calcium channels; desensitization; resensitization; β-adrenoceptor kinases; NG108-15 cells; patch clamp

Introduction

Opioid receptors, classified into at least three types (δ, μ and κ), mediate the effects of endogenous opioid peptides and opioid analgesics. At the cellular level, opioid receptors couple to adenylate cyclase, K^+ and Ca^{2+} channels and phospholipase C via pertussis toxin (PTX)-sensitive G proteins (Smart & Lambert, 1996). These opioid receptor signalling systems have been shown to undergo desensitization on continuous exposure to agonists in a variety of cells and tissues. For example, desensitization of the δ-opioid receptor-mediated inhibition of adenylate cyclase in NG108-15 neuroblastoma × glioma hybrid cells and the μ-opioid receptor-mediated activation of K^+ channels in rat locus coeruleus neurones has been investigated thoroughly, including the underlying cellular mechanisms (Law *et al*., 1982; Vachon *et al*., 1987; Harris & Williams, 1991; Fiorillo & Williams, 1996). Moreover, the involvement of protein kinases in opioid desensitization has been demonstrated in heterologous expression systems. Protein kinase C (PKC) has been shown to play a role in desensitization of the δ-opioid receptor-mediated phospholipase C activation in *Xenopus* oocytes (Ueda *et al*., 1995). Mestek *et al*. (1995) demonstrated the involvement of calcium/calmodulin-dependent protein kinase in desensitization of the μ-opioid receptor-K^+ channel coupling in *Xenopus* oocytes. Furthermore, overexpression of a dominant negative mutant of β-adrenoceptor kinase-1 (βARK1) attenuated desensitization of adenylate cyclase inhibition by the κ-opioid receptor in COS-7 cells and the δ-opioid receptor in HEK 293 cells (Raynor *et al*., 1994; Pei *et al*., 1995).

On the other hand, although desensitization of Ca^{2+} channel inhibition has been observed for the δ-opioid receptor in NG108-15 cells and the μ-opioid receptor in rat sensory neurones (McFadzean & Docherty, 1989; Nomura *et al*., 1994), the cellular and molecular mechanisms involved have been largely elusive. As a first step to approach this issue, we set out to investigate in detail the effects of prolonged application of [D-Ala^2, D-Leu^5]enkephalin (DADLE), a peptide opioid agonist, on Ca^{2+} channel currents in NG108-15 cells. NG108-15 cells, which possess a homogeneous population of the δ-opioid receptor (Evans *et al*., 1992), are suitable for this purpose because they have been established as a model system to study the acute and chronic effects of opioids. Moreover, foreign proteins can be stably expressed from exogenous cDNAs in NG108-15 cells (Fukuda *et al*., 1988; Morikawa *et al*., 1995), making them advantageous for the future study of the molecular mechanisms underlying desensitization. Experiments were designed to examine the time course, concentration-dependence and reversibility of desensitization. The possible involvement of protein kinases and phosphatases in desensitization and resensitization, respectively, was also tested.

Methods

Cell culture

NG108-15 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal

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bovine serum (FBS), 100 μM hypoxanthine, 0.4 μM aminopterine and 16 μM thymidine, as described previously (Morikawa *et al*., 1995). For electrophysiological recordings, cells were plated onto 15 mm diameter round glass coverslips coated with 0.01% (w/v) poly-L-lysine (Mol. Wt. >300,000) and differentiated for 5–14 days in DMEM supplemented with 1% FBS, 100 μM hypoxanthine, 16 μM thymidine and 1 mM dibutyryl cyclic AMP.

For PKC down-regulation, 1 μM phorbol 12-myristate 13-acetate (PMA) was added to the medium for 20–30 h (Gucker & Bidlack, 1992). Stock solution of PMA was made at 2 mM in ethanol.

Electrophysiological recordings

A glass coverslip on which differentiated cells were grown was transferred to the recording chamber ($\sim$200 μl) and continuously perfused at 1–2 ml min^{-1}.

Ba^{2+} currents (I_{Ba}) through voltage-gated Ca^{2+} channels were recorded at room temperature (22–25°C) by the whole-cell variation of the patch clamp technique with a single-electrode voltage-clamp amplifier (Axoclamp-2B; Axon Instruments, Foster City, CA, U.S.A.). Patch pipettes had resistances of 2–4 MΩ when filled with the internal solution of the following composition: 120 mM CsCl, 20 mM TEA-Cl, 10 mM EGTA-CsOH, 2 mM Mg-ATP, 0.2 mM Na-GTP, 50 u ml^{-1} creatine phosphokinase, 20 mM Na_2 creatine phosphate and 10 mM HEPES; pH was adjusted to 7.2 with CsOH. The external solution contained 10 mM $BaCl_2$, 145 mM NaCl, 5.5 mM CsCl, 2 mM $MgCl_2$, 10 mM glucose, 0.25 μM tetrodotoxin (TTX) and 10 mM HEPES; pH was adjusted to 7.4 with NaOH. Currents were low-pass filtered at 1 kHz (−3 dB) and digitized at 5 kHz. Data were stored and analysed by the pCLAMP software (Axon Instruments). Leakage and capacitative currents were subtracted by an on-line P/5 protocol.

I_{Ba} was elicited every 20 s by applying 100 ms voltage steps to 0 mV from a holding potential of −80 mV. I_{Ba} amplitude was measured at the time of the peak of the control current within 15 ms after the onset of the voltage step. I_{Ba} was confirmed to be blocked completely by 500 μM Cd^{2+}, a nonselective Ca^{2+} channel blocker ($n=7$), except for a small transient current observed in 2 cells which peaked at about 1 ms and inactivated completely within 5 ms. This small transient current might be the TTX-resistant Na^+ current (Weiss & Sidell, 1991). Correction for rundown of I_{Ba} was made as follows, assuming that the current amplitude declines linearly with time (Randall & Tsien, 1995). First, I_{Ba} amplitude was plotted against time. Then, the portion of the plot before application of DADLE and the portion in which the recovery following washout of DADLE had reached a steady-state phase (i.e., after rebound facilitation had subsided, see Results) were fitted to a straight line and extrapolated to the whole plot. Finally, the original plot was divided by this straight line to obtain a plot of normalized I_{Ba} amplitude. I_{Ba} inhibition was calculated as $(1-\text{normalized } I_{Ba})\times 100\%$ in all cases.

Drug application

DADLE was diluted in the external solution and applied by perfusion through the recording chamber. Heparin, Rp-cyclic AMPS, PKI (14-24)amide and okadaic acid were diluted in the internal solution and introduced directly into the cell through the whole-cell pipette. A stock solution of okadaic acid was made with dimethyl sulphoxide (DMSO). Final concentration of DMSO was 0.1%.

Data analysis

The time course of desensitization was fitted to a single exponential function by the Chebyshev fit method supported by the data analysis programme Clampfit in pCLAMP. The time course of resensitization and concentration-response relationships were fitted to a single exponential function and a logistic function, respectively, by use of the Levenberg-Marquardt algorithm implemented in the ORIGIN software (Microcal Software, Northampton, MA, U.S.A.).

Data are expressed as the mean ± s.e.mean. Statistical analysis was made with ANOVA followed by Bonferroni/Dunn procedure and considered significant when $P<0.05$.

Cloning of the βARK1 cDNA and its expression in COS-7 cells

To obtain the cDNA encoding rat βARK1, reverse transcription-polymerase chain reaction (RT-PCR) amplification was carried out with total RNA from rat cerebrum as the template. PCR primers used were CGCAAGCTTGCCGCCAC-CATGGCCGACCTGGAGGCGGTACTGGCC (forward) containing a *Hind*III site and CGCGGGGAATTCGGTTCA-GAGGCCGTTGGCACTGCCACGC (reverse) containing an *Eco*RI site, which were synthesized based on the rat βARK1 cDNA sequence (Arriza *et al*., 1992). The PCR product was cloned into pcDNA3 (Invitrogen, San Diego, CA, U.S.A.) containing the cytomegalovirus promoter to yield the expression plasmid, pcDNA3BARKI. COS-7 cells were transiently transfected with pcDNA3BARKI by the calcium phosphate method and harvested after 72 h incubation.

RNA blot hybridization analysis

Total RNA from NG108-15 cells and mouse brain was analysed as described previously (Fukuda *et al*., 1993). The hybridization probe used was a 2.1-kb *Hind*III/*Eco*RI fragment from pcDNA3BARKI. The probe was labelled with [α-^{32}P]-dCTP by the random primer method. Autoradiography was performed at −80°C for 10 days with an intensifying screen.

Immunoblot analysis

Immunoblot analysis was performed essentially as described previously (Fukuda *et al*., 1996). Briefly, cell lysates were electrophoresed on an SDS-10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Clear Blot Membrane-p; ATTO, Tokyo, Japan). The membrane was blocked with 5% non-fat dry milk and incubated with anti-GRK2 (βARK1) antibody at 0.1 μg ml^{-1}. The blots were visualized by incubating with the horseradish peroxidase-conjugated secondary antibody followed by detection by the ECL system (Amersham, Buckinghamshire, U.K.).

Materials

DMEM was purchased from GIBCO (Grand Island, NY, U.S.A.), and hypoxanthine, aminopterine, thymidine and FBS from Dainippon Pharmaceutical (Osaka, Japan). DADLE was obtained from Peptide Institute (Osaka, Japan). PKI (14–24)amide was from Peninsula Laboratories (Belmont, CA, U.S.A.). [α-^{32}P]-dCTP was purchased from Amersham (Tokyo, Japan). Anti-GRK2 (βARK1) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other chemicals were purchased from Wako Pure Chemical

Industries (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO, U.S.A.).

Results

Application of 100 nM DADLE rapidly elicited inhibition of I_{Ba} in differentiated NG108-15 cells, which is mediated by the δ-opioid receptor (Morikawa *et al*., 1995). The inhibition declined (or desensitized) with continued perfusion of DADLE (15–30 min) to reach a sustained, steady-state phase (Figure 1). The initial peak inhibition and the sustained inhibition of I_{Ba} by 100 nM DADLE were $40\pm3\%$ and $18\pm2\%$, respectively ($n=36$). The magnitude and time constant of desensitization were $57\pm3\%$ and 4.4 ± 0.6 min, respectively ($n=36$). I_{Ba} amplitude immediately after washout of DADLE usually exceeded the control level (rebound facilitation) and then gradually faded in 2–20 min (Figure 1), as found by Kasai (1991).

Concentration-response relationships

To characterize the concentration-response relationships of desensitization, prolonged application of DADLE was made at various concentrations. Desensitization was observed whenever measurable I_{Ba} inhibition was elicited, regardless of the concentration of DADLE perfused. There was no clear concentration-dependence for the magnitude and time constant of desensitization (data not shown). The concentration-response relationships for the peak and sustained I_{Ba} inhibition are shown in Figure 2. By fitting the concentration-response curve for the peak inhibition to a logistic function, the EC_{50} value and maximal inhibition were estimated to be 3.7 nM and 41%, respectively. Maximal peak inhibition was attained by 100 nM DADLE, whereas the sustained inhibition by DADLE was maximal at 10 nM.

Next, the degree of DADLE-induced desensitization of the δ-opioid receptor-Ca^{2+} channel coupling was assessed, by calculating the ratio of peak I_{Ba} inhibition by 1 μM DADLE before and after steady-state desensitization had been induced by various concentrations ($\leqslant 1$ μM) of DADLE (Figure 3a). The concentration-response curve for the degree of desensitization thus obtained is shown in Figure 3b. We confirmed that maximal desensitization was attained with 1 μM DADLE, as 10 μM DADLE produced no further desensitization after 1 μM DADLE had induced steady-state desensitization ($n=5$, Figure 3c). The EC_{50} value for desensitization was estimated to be 78 nM when the curve was fitted to a logistic function.

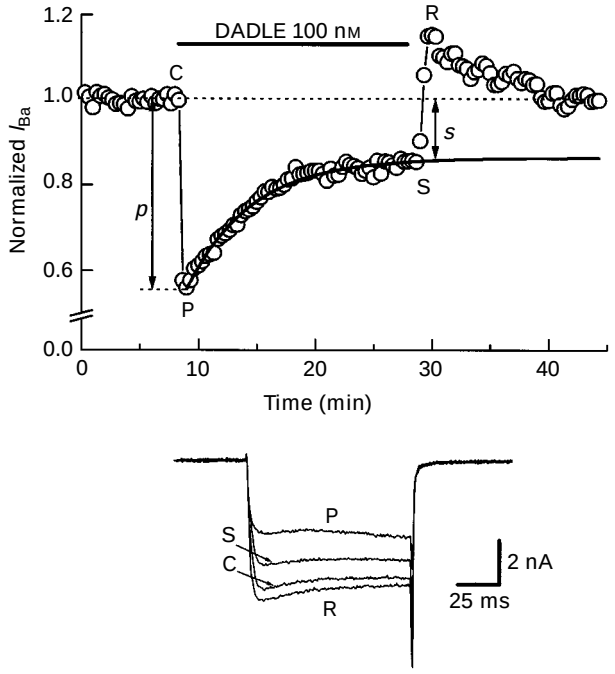

Figure 1 The time course of desensitization of DADLE-induced I_{Ba} inhibition. Normalized I_{Ba} amplitude was plotted versus time. Current amplitudes were normalized to the control amplitude after correction for rundown of I_{Ba} (see Methods for details). DADLE (100 nM) was perfused at the time indicated by the horizontal bar. The smooth line represents a fit to a single exponential function. Peak and sustained inhibition of I_{Ba} were defined as $p\times100$ (%) and $s\times100$ (%), respectively, as indicated in the figure. The magnitude of desensitization was defined as $\{(p-s)/p\}\times100$ (%). Peak and sustained inhibition were 36% and 21%, respectively, while the magnitude and time constant of desensitization were 65% and 5.4 min, respectively, in this cell. Traces of I_{Ba} representing control (C), peak inhibition (P), sustained inhibition (S) and rebound facilitation (R) are shown below.

Expression of βARK in NG108-15 cells

As βARK has been implicated in desensitization of other opioid receptor systems (Raynor *et al*., 1994; Pei *et al*., 1995), we attempted to clarify the expression of βARK in NG108-15 cells by RNA blot hybridization analysis and immunoblot analysis. NG108-15 cells, as well as mouse brain, contained an RNA species of $\sim$4 kb which can be hybridized with the βARK1 cDNA probe (Figure 4a). Furthermore, when probed with anti-βARK1 antibody, the extract from NG108-15 cells exhibited a protein with an electrophoretic mobility similar to the protein expressed from the rat βARK1 cDNA in COS-7 cells, which migrates with an apparent M_r of $\sim$83 kDa (Figure 4b). These results suggest that βARK1 is endogenously expressed in NG108-15 cells.

Involvement of βARK, but neither PKA nor PKC, in desensitization of the opioid-induced inhibition of Ca^{2+} channel currents

We next investigated the possible involvement of protein kinases in desensitization of DADLE-induced I_{Ba} inhibition by

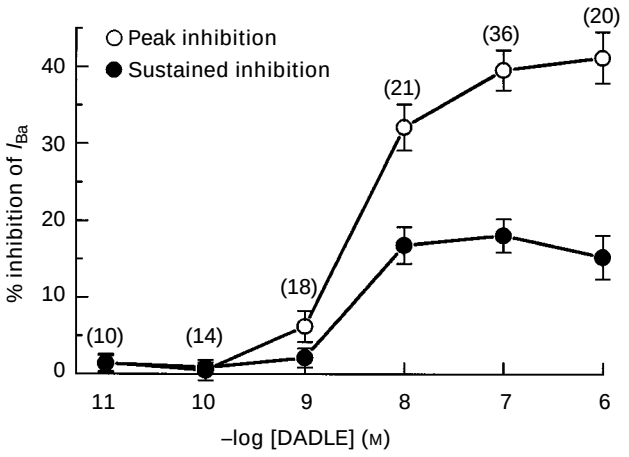

Figure 2 Concentration-response curves for the peak and sustained inhibition of I_{Ba} by continuous application of DADLE. Each point represents the mean for the number of cells indicated in the parentheses; vertical lines show s.e.mean.

means of pharmacological manipulations. We first applied heparin, which is known to inhibit βARK activity (Lohse *et al.*, 1990), at a concentration of 250 μg ml^{-1} by intracellular dialysis through the whole-cell pipette. The magnitude of desensitization of the response to 100 nM DADLE was significantly attenuated and the time constant of desensitization was also significantly prolonged (Figure 5, Table 1). In contrast, the magnitude and time constant of desensitization were not significantly affected by intracellular dialysis of 200 μM Rp-cyclic AMPS, a competitive inhibitor of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) for binding to cyclic AMP-dependent protein kinase (PKA) (Rothermel & Parker Botelho, 1988), or 20 μM PKI(14–24)amide, a specific PKA inhibitory peptide (Glass *et al.*, 1989) (Table 1). Furthermore, 20–30 h pretreatment of the cells with 1 μM PMA, which is thought to induce down-regulation of PKC in NG108-15 cells (Gucker & Bidlack, 1992), or its inactive analogue, 4α-PMA, had no significant effect on desensitization (Table 1). None of these treatments had a significant effect on the peak inhibition of I_{Ba} (Table 1). Taken together, these data suggest that βARK, but not PKA nor PKC, participates in desensitization of DADLE-induced I_{Ba} inhibition.

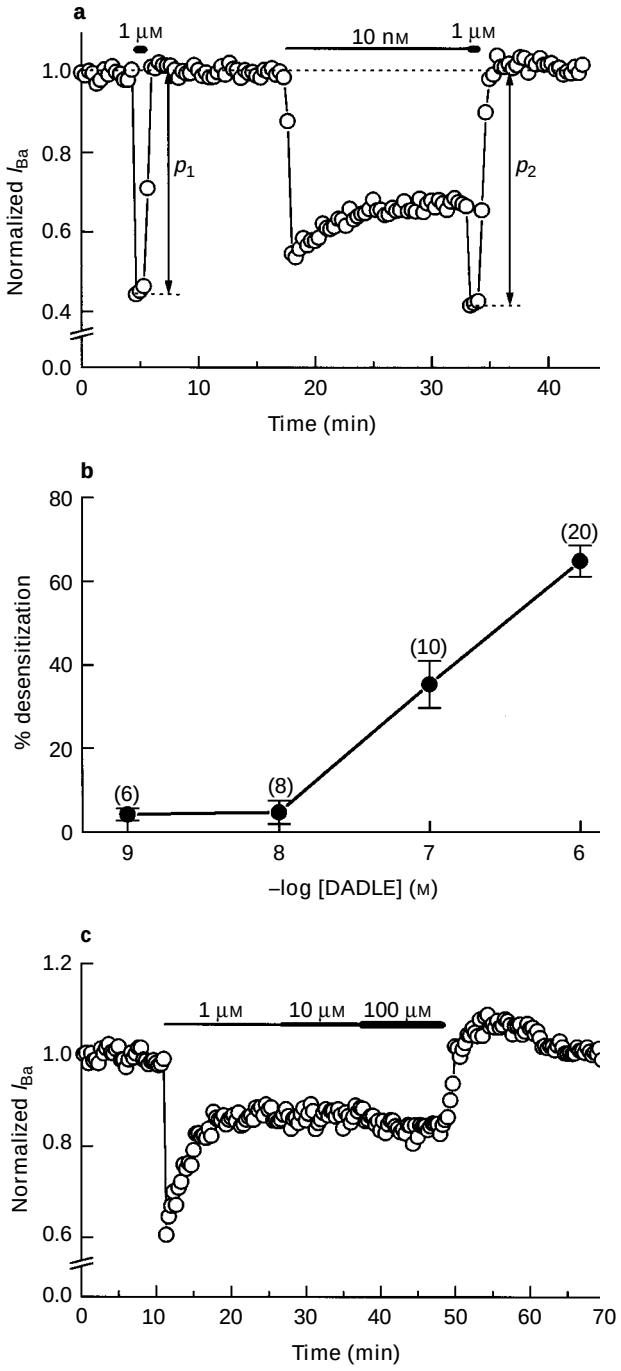

Figure 3 Concentration-dependence of DADLE-induced desensitization of the δ-opioid receptor-Ca^{2+} channel coupling. (a) Plot of normalized I_{Ba} amplitude versus time. Initially, brief application (1 min) of 1 μM DADLE was made to elicit the control, undesensitized inhibition (p_1). After washout, 0–100 nM DADLE (10 nM in this figure) was continuously perfused to induce steady-state desensitization. Then, 1 μM DADLE was applied again to elicit the second, desensitized inhibition (p_2). Percentage desensitization was calculated as $\{(p_1-p_2)/p_1\}\times 100$ (%). (b) Concentration-response curve for desensitization. The concentration of DADLE used to induce steady-state desensitization is plotted on the abscissa scale. Each point represents the mean for the number of cells indicated in the parentheses and vertical lines show s.e.mean. For 1 μM DADLE, percentage desensitization was calculated as in Figure 1 by a single perfusion which induced steady-state desensitization. (c) Plot of normalized I_{Ba} amplitude versus time. Sequential application of 1, 10 and 100 μM DADLE was made as indicated by the horizontal bars.

Resensitization of the opioid-induced inhibition of Ca^{2+} channel currents

The response to DADLE recovered (resensitized) after washout of DADLE (Figure 6a). The time course of resensitization was determined by varying the length of the DADLE-free interval. The peak inhibition of I_{Ba} induced by a second application of DADLE gradually recovered as the DADLE-free interval was prolonged. Resensitization was nearly complete in 15–20 min. The time course of resensitization could be fitted to a single exponential function with a time constant of 6.7 min (Figure 6b).

If desensitization involves phosphorylation by βARK, which is a serine/threonine kinase, resensitization must reflect a process of dephosphorylation. To test for the possible involvement of serine/threonine phosphatases in resensitization, we examined the effect of okadaic acid, an inhibitor of serine/threonine phosphatases 1 and 2A (Bialojan & Takai, 1988). Inclusion of 1 μM okadaic acid in the whole-cell pipette significantly attenuated the degree of resensitization after a DADLE-free interval of 15 min ($57\pm10\%$, $n=8$ vs $93\pm3\%$, $n=7$ for control, $P<0.01$), whereas solvent alone (0.1% DMSO) had no significant effect ($90\pm6\%$, $n=5$). They did not significantly affect the peak inhibition of I_{Ba} nor the magnitude and time constant of desensitization (Table 1).

Discussion

In the present experiments, desensitization of the δ-opioid receptor-mediated inhibition of Ca^{2+} channel currents was studied in detail in NG108-15 cells. The response to 100 nM DADLE desensitized by 57% with a time constant of 4.4 min. The magnitude and rate of desensitization were comparable to those obtained for desensitization of the μ-opioid receptor-mediated Ca^{2+} channel inhibition in rat sensory neurones, which was 45% at 5 min (Nomura *et al.*, 1994). In contrast, desensitization of the δ-opioid receptor-mediated inhibition of adenylate cyclase has been shown to proceed in the time frame of hours in NG108-15 cells (Law *et al.*, 1982; Vachon *et al.*, 1987). It may be that some different mechanisms are involved in these two types of desensitization in NG108-15 cells.

The response to DADLE consistently showed desensitization whenever there was a measurable inhibition, even with low nanomolar concentrations. However, the EC_{50} for desensitization (78 nM) was more than one order of magnitude

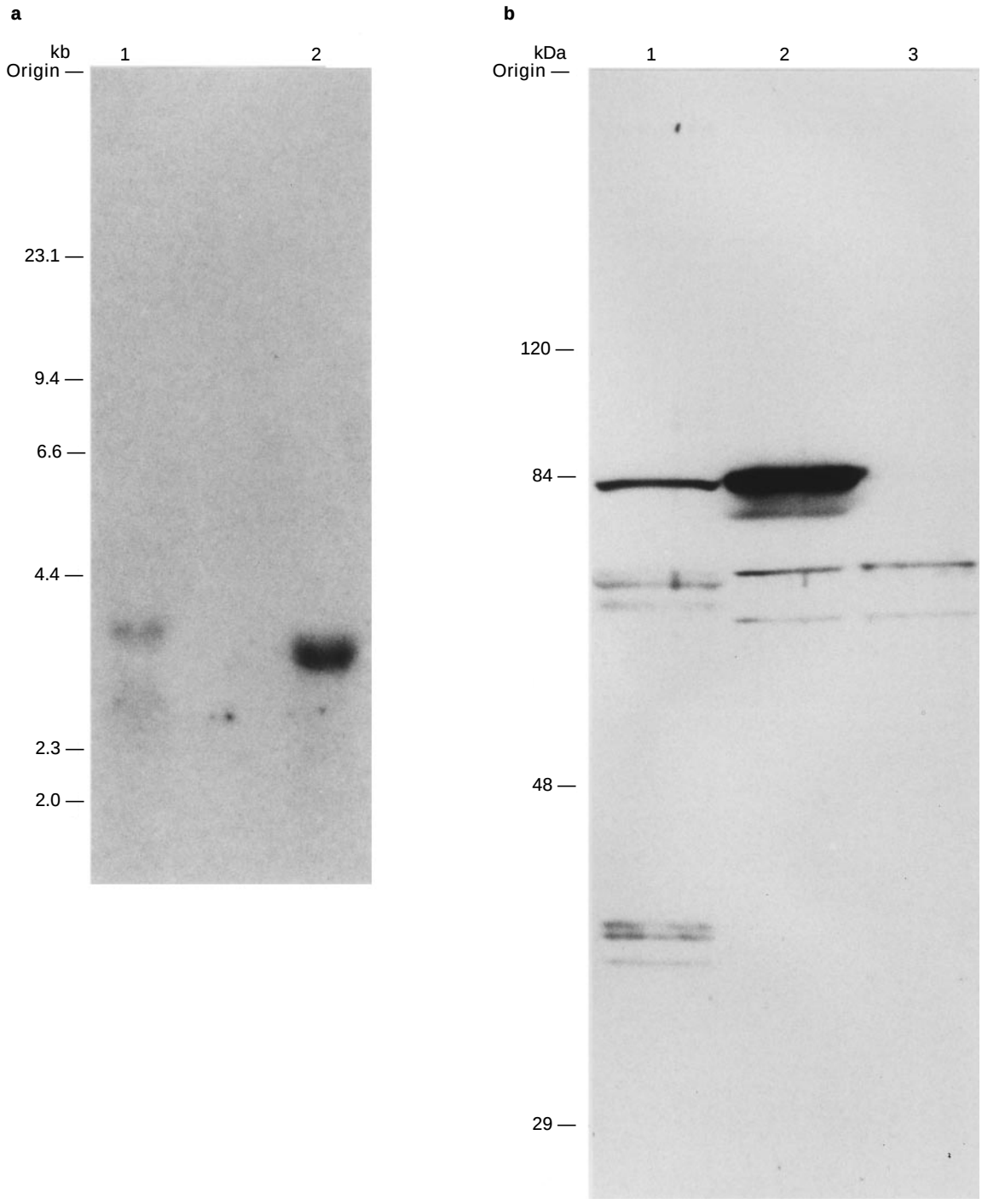

Figure 4 Expression of βARK1 in NG108-15 cells. (a) Total RNA preparations from NG108-15 cells (50 μg, lane 1) and mouse brain (5 μg, lane 2) were electrophoresed, blotted and hybridized with radiolabelled βARK1 cDNA probe. The size markers are indicated. (b) Extracts from NG108-15 cells (lane 1), COS-7 cells transfected with rat βARK1 cDNA (lane 2) and non-transfected COS-7 cells (lane 3) were probed with an antibody raised against the carboxyl terminus of βARK1. The positions of molecular weight standards are indicated. Data are representative of three separate experiments.

larger than that for Ca^{2+} channel inhibition (3.7 nM). Furthermore, while 100 nM DADLE produced maximal inhibition of Ca^{2+} channel currents, a 10 fold higher concentration of DADLE (1 μM) was necessary to induce maximal desensitization. In line with this, it has been shown that the concentration of agonists required to cause maximal desensitization is larger than that needed to saturate the signal transduction pathway for the μ-opioid receptor/α_2-adrenoceptor-K^+ channel coupling in rat locus coeruleus neurones and the α_2-adrenoceptor-Ca^{2+} channel coupling in embryonic chick sensory neurones (Fiorillo & Williams, 1996; Diversé-Pierluissi *et al*., 1996).

Desensitization of neurotransmitter-induced Ca^{2+} channel inhibition has been shown to be resistant to reversal in rat and frog sympathetic neurones and rat sensory neurones (Ikeda & Schofield, 1989; Elmslie, 1992; Nomura *et al*., 1994). Desensitization was reversible in our case however, and the time course of resensitization could be fitted to a single exponential function with a time constant of 6.7 min. Moreover, desensitization could be reproduced after resensi-

tization had occurred (Figure 6a), further reinforcing the reversibility of desensitization.

Desensitization is a common feature observed in the signal transduction of G protein-coupled receptors. It has been most extensively investigated in the β-adrenoceptor system, where receptor phosphorylation by βARK and PKA leads to desensitization (Lohse *et al*., 1990), while desensitization of the δ-opioid receptor system has been shown to involve βARK and PKC (Pei *et al*., 1995; Ueda *et al*., 1995). Indeed, we could detect the expression of βARK1 in NG108-15 cells by RNA blot hybridization analysis and immunoblot analysis, and heparin, an inhibitor of βARK, significantly attenuated the magnitude and rate of desensitization. On the other hand, both inhibition of PKA by Rp-cyclic AMPS or PKI (14–24)amide and down-regulation of PKC by long-term PMA treatment failed to affect desensitization. These results suggest that PKA and PKC does not play a role in desensitization, although activation of the δ-opioid receptor has been shown to stimulate phospholipase C, and subsequently PKC, in NG108-15 cells (Smart & Lambert, 1996). In addition, it was suggested that serine/threonine phosphatases mediate resensitization, as okadaic acid significantly attenuated the degree of resensitization. Thus, desensitization and resensitization may reflect the reversible processes of phosphorylation and dephosphorylation of the substrate protein(s), respectively.

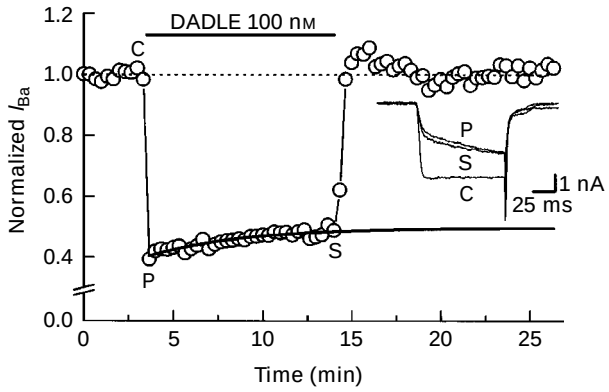

Figure 5 Effect of heparin on desensitization of DADLE-induced I_{Ba} inhibition. Plot of normalized I_{Ba} amplitude versus time in a cell dialyzed with 250 μg ml^{-1} heparin through the whole-cell pipette. DADLE (100 nM) was perfused at the time indicated by the horizontal bar. The smooth line represents a fit to a single exponential function. The response desensitized by 16% with a time constant of 6.5 min in this cell. Traces of I_{Ba} representing control (C), peak inhibition (P) and sustained inhibition (S) are shown in the inset.

Recently, Diversé-Pierluissi *et al*. (1996) demonstrated that βARK2, but not βARK1, is specifically involved in desensitization of the α_2-adrenoceptor-mediated Ca^{2+} channel

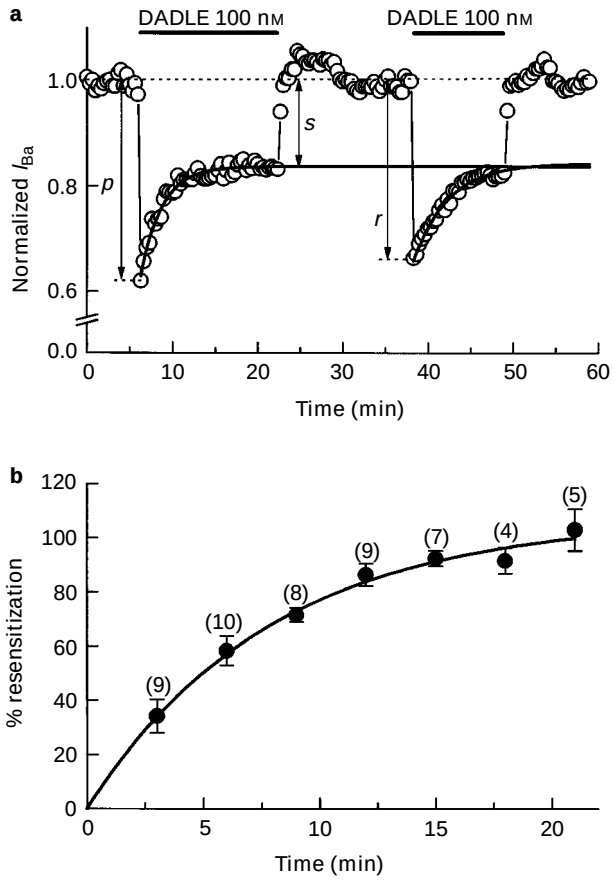

Figure 6 Resensitization of DADLE-induced I_{Ba} inhibition. (a) Plot of normalized I_{Ba} amplitude versus time. Initially a continuous perfusion of 100 nM DADLE was made to obtain the peak (*p*) and sustained (*s*) inhibition. After a DADLE-free interval of 3–21 min (15 min in this figure), with an extra 40 s to allow for the washout of DADLE, the second perfusion of 100 nM DADLE was made to elicit a second, resensitized inhibition (*r*). Percentage resensitization was defined as $\{(r-s)/(p-s)\} \times 100$ (%). In the experiment shown, the second perfusion was prolonged and induced desensitization comparable to the first perfusion. The smooth lines represent fits to a single exponential function. (b) The time course of resensitization. Percentage resensitization was plotted as a function of DADLE-free interval. Each point represents the mean for the number of cells indicated in the parentheses and vertical lines show s.e.mean. The smooth line is a fit to a single exponential function.

Table 1 Effects of protein kinase and phosphatase inhibitors on DADLE-induced I_{Ba} inhibition and its desensitization

Treatment	*Peak inhibition* (%)	*Sustained inhibition* (%)	*Desensitization* (%)	*Time constant of desensitization* (min)
Control	40±3 (36)	18±2 (36)	57±3 (36)	4.4±0.6 (36)
Heparin (250 μg ml^{-1})	41±3 (15)	30±3 (15)*	28±6 (15)*	9.0±2.7 (12)*
Rp-cyclicAMPS (200 μM)	47±3 (14)	19±2 (14)	59±5 (14)	5.7±0.8 (14)
PKI (14–24) amide (20 μM)	36±4 (8)	12±2 (8)	66±4 (8)	6.0±0.9 (8)
PMA (1 μM; 20–30 h)	33±3 (11)	13±1 (11)	59±4 (11)	6.1±0.9 (11)
4α-PMA (1 μM; 20–30 h)	41±4 (7)	19±5 (7)	58±7 (7)	6.4±0.9 (7)
Okadaic acid (1 μM)	33±4 (11)	17±3 (11)	52±6 (11)	4.2±0.8 (11)
DMSO (0.1%)	37±6 (5)	12±1 (5)	64±5 (5)	4.0±0.8 (5)

All drugs were included in the whole-cell pipette, except PMA and 4α-PMA, which were added to the culture medium for 20–30 h before experiments. Peak and sustained inhibition of I_{Ba} and the magnitude and time constant of desensitization induced by 100 nM DADLE were calculated for each cell as described in the legend to Figure 1. The time constant of desensitization was not determined for 3 cells with heparin in the whole-cell pipette due to a very small degree of desensitization. Data are expressed as the mean±s.e.mean for the number of cells indicated in the parentheses. *$P<0.01$, significantly different from control.

inhibition in embryonic chick sensory neurones. It will be of interest to test whether specificity exists as well between the subtypes of βARK and opioid desensitization in NG108-15 cells.

Rebound facilitation of Ca^{2+} channel currents was frequently observed upon washout of DADLE. It has been attributed to the reversal of tonic Ca^{2+} channel inhibition by constitutively active G proteins in NG108-15 cells (Kasai, 1991). Thus, it can be viewed as a manifestation of G protein desensitization during continuous δ-opioid receptor activation. However, desensitization and rebound facilitation appeared to be two distinct processes, as rebound facilitation could be observed even after brief application of DADLE, which induced little, if any, desensitization (data not shown).

Neuropeptides released from nerve terminals persist in the extracellular fluid for several minutes (Scheller & Hall, 1992). Also, it has been shown that the extracellular concentration of methionine-enkephalin, an endogenous opioid peptide acting on μ- and δ-opioid receptors, reaches micromolar levels (Kendrick, 1990; Hashizume *et al*., 1994). Therefore, it is likely that desensitization of the opioid-induced Ca^{2+} channel inhibition described here plays a role in controlling the physiological actions of endogenous opioid peptides.

Meanwhile, opioid desensitization has been argued to represent a cellular model for opioid tolerance (Nestler *et al*., 1993). In this regard, it has been found that the opioid receptor signalling is desensitized following chronic morphine treatment of rats (Christie *et al*., 1987; Noble & Cox, 1996). Moreover, we made observations that desensitization induced by lower concentrations of DADLE can be overcome by higher concentrations (data not shown), which resembles the clinical experience in that increasing the dosage of opioid analgesics is effective for the patients who have developed opioid tolerance. Opioid desensitization might be an initial cellular adaptive mechanism eventually leading to opioid tolerance, which develops over days to weeks.

In conclusion, the δ-opioid receptor-mediated Ca^{2+} channel inhibition desensitizes by more than 50% in the time frame of minutes in NG108-15 cells. This desensitization is sensitive to the concentration of the agonist at a concentration range where maximal Ca^{2+} channel inhibition is attained. Our data show that the desensitization is a reversible process, which is suggested to reflect phosphorylation and dephosphorylation mediated by βARK and serine/threonine phosphatases, respectively.

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