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Inhibitory effect of 2,3-butanedione monoxime (BDM) on Na^+/Ca^{2+} exchange current in guinea-pig cardiac ventricular myocytes

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1 The effect of 2,3-butanedione monoxime (BDM), a 'chemical phosphatase', on Na^+/Ca^{2+} exchange current (I_{NCX}) was investigated using the whole-cell voltage-clamp technique in single guinea-pig cardiac ventricular myocytes and in CCL39 fibroblast cells expressing canine NCX1.

2 I_{NCX} was identified as a current sensitive to KB-R7943, a relatively selective NCX inhibitor, at 140 mM Na^+ and 2 mM Ca^{2+} in the external solution and 20 mM Na^+ and 433 nM free Ca^{2+} in the pipette solution.

3 In guinea-pig ventricular cells, BDM inhibited I_{NCX} in a concentration-dependent manner. The IC_{50} value was 2.4 mM with a Hill coefficients of 1. The average time for 50% inhibition by 10 mM BDM was 124 ± 31 s ($n = 5$).

4 The effect of BDM was not affected by 1 μM okadaic acid in the pipette solution, indicating that the inhibition was not *via* activation of okadaic acid-sensitive protein phosphatases.

5 Intracellular trypsin treatment *via* the pipette solution significantly suppressed the inhibitory effect of BDM, implicating an intracellular site of action of BDM.

6 PAM (pralidoxime), another oxime compound, also inhibited I_{NCX} in a manner similar to BDM.

7 Isoprenaline at 50 μM and phorbol 12-myristate 13-acetate (PMA) at 8 μM did not reverse the inhibition of I_{NCX} by BDM.

8 BDM inhibited I_{NCX} in CCL39 cells expressing NCX1 and in its mutant in which its three major phosphorylatable serine residues were replaced with alanines.

9 We conclude that BDM inhibits I_{NCX} but the mechanism of inhibition is not by dephosphorylation of the Na^+/Ca^{2+} exchanger as a 'chemical phosphatase'.

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Abbreviations: BDM, 2,3-butanedione monoxime; DIDS, 4,4′-dinitrostilbene-2,2′-disulphonic acid disodium salt; DMSO, dimethylsulphoxide; HPLC, high performance liquid chromatography; I_{NCX}, Na^+/Ca^{2+} exchange current; I–V curve, current–voltage relation curve; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate; NCX1, canine cardiac Na^+/Ca^{2+} exchanger; PAM, pralidoxime; PMA, phorbol 12-myristate 13-acetate; PP1, proteinphosphatase 1; PP2A, proteinphosphatase 2A

Introduction

Na^+/Ca^{2+} exchange is one of the major mechanisms regulating intracellular Ca^{2+} concentration in cardiac myocytes (for review see Blaustein & Lederer, 1999; Philipson & Nicoll, 2000). In normal cardiac myocytes, the electrogenic Na^+/Ca^{2+} exchanger extrudes Ca^{2+} to maintain an intracellular Ca^{2+} concentration which is 10^3–10^4 times lower than that in the exterior. Under ischaemic conditions, Ca^{2+} overload and reperfusion-induced arrhythmia are well known pathological phenomena in which the involvement of Na^+/Ca^{2+} exchange current (I_{NCX}) has been suggested (reviewed by Ch'en *et al.*, 1998). Therefore, understanding the characteristics of this transporter is crucial in physiological and pathological cardiac conditions. I_{NCX} can be readily recorded under the whole-cell voltage clamp by loading Na^+ and Ca^{2+} in internal and external solutions (Kimura *et al.*, 1986; 1987; 1999). Recently, a relatively selective Na^+/Ca^{2+} exchange inhibitor, KB-R7943, has been developed and it can be used to identify I_{NCX} (Watano *et al.*, 1996; Iwamoto *et al.*, 1996a; Kimura *et al.*, 1999).

BDM (2,3-butanedione monoxime) is a well known agent which suppresses cardiac muscle contraction (Bergrey *et al.*, 1981; Li *et al.*, 1984; West & Stephenson, 1989; Watanabe *et al.*, 1996). If BDM does not affect the exchanger and allows

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the loading of a high Ca^{2+} concentration in cardiac myocytes while suppressing contraction, it will become a useful tool for investigating the inward I_{NCX} or Ca^{2+} efflux mode of the exchanger. However, in a preliminary experiment, we found that BDM inhibited I_{NCX}. Since only few inhibitors of I_{NCX} have been reported, we investigated BDM as a new inhibitor of the Na^+/Ca^{2+} exchanger.

BDM was originally developed to counteract organophosphorus poisoning of acetylcholinesterase. It re-activates the enzyme by binding and removing the phosphate group from the inactivated phosphorylated enzyme. Therefore, the drug was considered to possess a phosphatase-like activity and has been called a 'chemical phosphatase' (Holmstedt, 1959). Besides suppressing cardiac muscle contraction, BDM has multiple effects in a variety of tissues, such as suppressing contraction of skeletal (Fryer *et al*., 1988) and smooth muscles (Österman *et al*., 1993), inhibiting voltage-gated Ca^{2+} channels (Coulombe *et al*., 1990; Chapman, 1993; Ferreira *et al*., 1997) and the transient outward K^+ channels in cardiac myocytes (Coulombe *et al*., 1990), smooth muscles (Langer & Paul, 1991) and neurones (Huang & McArdle, 1992). Some of these effects were attributed to the dephosphorylation activity of BDM, but Allen *et al*. (1998) demonstrated that BDM inhibited mutant Ca^{2+} channels in *Xenopus* oocytes which lacked protein kinase A-dependent phosphorylation sites and thus it is unlikely that a phosphatase action of BDM is involved.

There are controversial reports on the effect of phosphorylation of the mammalian Na^+/Ca^{2+} exchanger. In NCX transfected fibroblast cells, the activation of protein kinase C by PMA augmented the NCX activity (Iwamoto *et al*., 1998). However, no trace of phosphorylation was found in CHO cells transfected with NCX (Condrescu *et al*., 1995). In this study, using the whole-cell voltage clamp, we examined the effect of BDM on I_{NCX} and its mechanism in single guinea-pig ventricular cells and CCL39 fibroblast cells stably expressing dog heart NCX1.

Methods

Isolation of cells

All experiments were performed according to the regulations of the Animal Research Committee of Fukushima Medical University. Single cardiac ventricular cells were isolated by the following method. Guinea-pigs weighing 250–400 g were anaesthetized by intraperitoneal injection of pentobarbital (30 mg kg^{-1}). The chest was opened under artificial ventilation, the aorta was cannulated *in situ*, and the heart was removed. After washing out the blood with Tyrode solution, the heart was mounted in a Langendorff perfusion system. Tyrode solution contained (mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.33, glucose 5.5 and HEPES (4, (2-hydroxyethyl)-1-piperazine-ethanesulphonic acid)-NaOH 5 (pH 7.4). The perfusate was changed to Ca^{2+}-free Tyrode solution to stop the heartbeat and then to one containing 0.01% w v^{-1} collagenase (Wako, Osaka, Japan) and 0.002% w v^{-1} alkaline protease (Nagase, Tokyo, Japan). After about 20 min, the collagenase solution was washed out by perfusing with a high K^+, low Cl^- solution (modified KB solution; Isenberg & Klockner, 1982; Nakaya *et al*., 1993). The modified KB solution contained (mM): KOH 70, 1-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, EGTA 0.2 and HEPES-KOH buffer 10 (pH 7.2). Incisions were made in cardiac ventricular tissue in the modified KB solution and the tissue was shaken gently to isolate the cells. The cell suspension was stored at 4°C for later use.

Cell cultures

CCL39 cells (American Type Culture Collection) untransfected and those transfected with NCX1 were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 7.5% heat-inactivated foetal calf serum, 50 units ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. Two types of stably expressing NCX1-CCL39 cells were used. One was transfected with a wild type NCX1 and the other with a mutant NCX1 in which three serine residues are replaced by alanine; i.e. S249A/S250A/S357A (Iwamoto *et al*., 1998). The cells were cultured for 2 or 3 days on small pieces (2×8 mm) of cover glasses and used for the whole cell voltage clamp experiments.

Patch-clamp recording

Membrane currents were recorded by the whole-cell patch-clamp method. Single cardiac ventricular cells were placed in a recording chamber (1 ml volume) attached to an inverted microscope (Model 801212, Nikon, Tokyo, Japan) and superfused with the HEPES-Tyrode solution at a rate of 5 ml min^{-1}. The temperature of the bath solution was maintained at 36 ± 0.5°C with a water jacket. Patch pipettes were forged from 1.5 mm diameter glass capillaries (Nihon Rikagaku Kikai, Tokyo) with a microelectrode puller (pp-83, Narishige, Tokyo, Japan). The pipette resistance was 2–4 MΩ when filled with the pipette solution. The pipette solution contained (mM): NaCl 20, BAPTA (1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid) 20, $CaCl_2$ 13 (free Ca^{2+} concentration, 433 nM), CsOH 120, Aspartic acid 50, $MgCl_2$ 3, MgATP 5 and HEPES 20 (pH 7.2 with aspartic acid). The free Ca^{2+} concentration was calculated by using the equations of Fabiato & Fabiato (1979) modified with binding constants of BAPTA instead of EGTA. The extracellular solution contained (mM): NaCl 140, $CaCl_2$ 1, $MgCl_2$ 1, ouabain 0.02, nifedipine 0.004, ryanodine 0.005 and HEPES 5 (pH 7.2 with CsOH). The electrode was connected to a patch-clamp amplifier (TM-1000, Act ME, Tokyo, Japan). Recording signals were filtered at 2.5 kHz bandwidth, and the series resistance was compensated. Current signals were stored on-line and analysed by a computer (PC-9801RX, NEC, Tokyo, Japan).

The calculated reversal potential of the exchange current was −68.4 mV at 3 Na^+: 1 Ca^{2+} stoichiometry with the ionic conditions used. The average reversal potential of the KB-R7943-sensitive current was -60 ± 3 mV ($n = 10$), which coincided with the holding potential. The reversal potential of I_{NCX} (E_{NCX}) typically falls toward the holding potential due to the movement of Na^+ and Ca^{2+} by Na^+/Ca^{2+} exchange (Yasui & Kimura, 1990). Ramp pulses of 500 ms duration were given with 10 s intervals. The ramp pulse initially depolarized from −60 to +60 mV, then hyperpolarized to −110 mV and depolarized back to the holding

potential at a speed of 680 mV s^{-1}. The descending limb of the ramp was used to plot the I–V curve without capacitative current compensation. Ca^{2+} current, K^+ currents, Na^+-K^+ pump current and Ca^{2+} release channels of the sarcoplasmic reticulum were blocked by nifedipine, Cs^+, ouabain and ryanodine in the external solution, respectively.

Drugs

BDM (2,3-butanedione monoxime), DIDS (4,4′-dinitrostilbene-2,2′-disulphonic acid disodium salt), nifedipine, ouabain, PAM (pralidoxime), PMA (phorbol 12-myristate 13-acetate) and BAPTA were purchased from Sigma Chemical Co. (St Louis, U.S.A.), ryanodine from AgriSystems International (Wind Gap, Pennsylvania, U.S.A.), okadaic acid from RBI (MA, U.S.A.) and trypsin from Difco Laboratories (Detroit, MI, U.S.A.). KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]-ethyl]isothiourea methanesulphonate) was a kind gift from Kanebo Co. Ltd. (Osaka, Japan).

BDM was directly dissolved in the extracellular solution. Nifedipine and KB-R7943 were first dissolved in dimethylsulphoxide (DMSO) and added to extracellular solutions with a final concentration of DMSO $\leqslant$ 0.1% which did not affect I_{NCX}. Okadaic acid (1 μM) and trypsin (2.5 $\sim$5 μg ml^{-1}) were directly dissolved in the pipette solution. All the chemicals used were the highest grade available.

HPLC

Since BDM is known as a chemical phosphatase, whether BDM dephosphorylates ATP and converts ATP into ADP and/or other metabolites was examined. BDM at 10 mM was mixed with 2.2 mM ATP for 5$\sim$10 min in a glass tube, and a sample of the mixture was analysed by high performance liquid chromatography (HPLC) (BIP-I, Japan Spectroscopic Co. Ltd) as described previously (Matsuoka *et al*., 1995). ATP and its metabolites were monitored at 258 nm with a UV detector (UVIDEC-100, Japan Spectroscopic Co. Ltd).

Data analysis

All the values are presented as means $\pm$ s.e.mean (number of experiments). Student's *t*-test and analysis of variance were used for the statistical analyses. *P* values of less than 0.05 were considered to be significant. The concentration-response data were fitted and IC_{50} and Hill coefficient values were obtained using Delta Graph Professional (Polaroid Computing, Tokyo, Japan) on a Macintosh computer (Apple Computer, Cupertino, CA, U.S.A.).

The percentages of inhibition of I_{NCX} at 50 mV by various concentrations of BDM were fitted by the logistic equation:

$$\text{Per cent inhibition} = 100 \times 1/\{1 + (IC_{50}/[D])^{nH}\}$$

where [D] indicates the concentration of BDM, IC_{50} the concentration of the drug for half-maximum inhibition and n_H is an empirical parameter describing the steepness of the fit which has the same meaning as the Hill coefficient.

Results

Effects of BDM on I_{NCX}

Figure 1A shows a typical chart recording of the current in control external solution under the whole-cell voltage clamp.

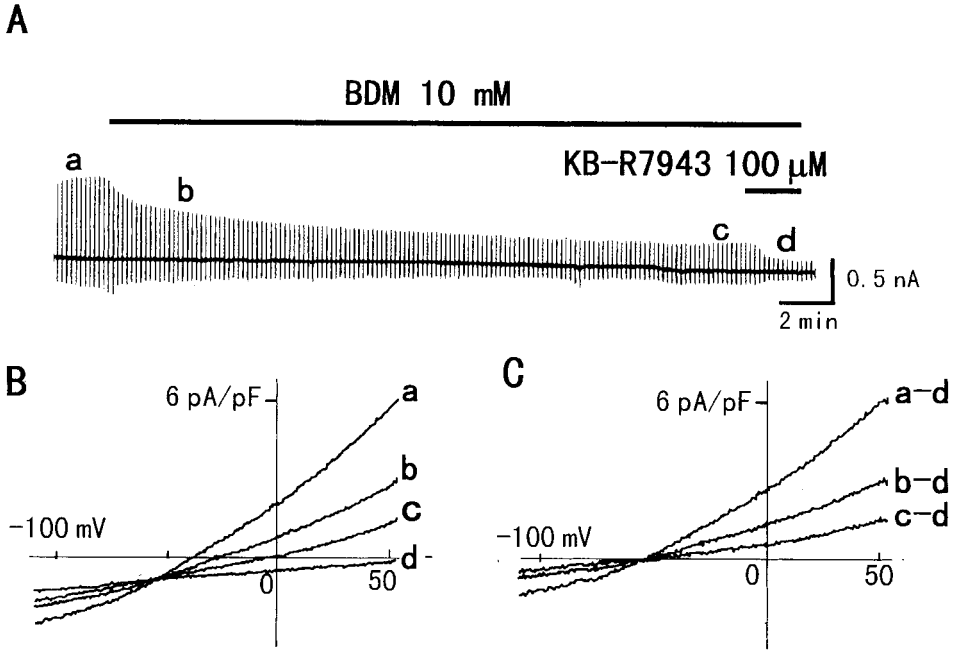

Figure 1 Effect of BDM on I_{NCX}. (A) Chart recording of the membrane current. The horizontal bars above the chart indicate where 10 mM BDM and 100 μM KB-R7943 were applied externally. (B) I–V curves obtained at the corresponding labels in (A). a is control, b at 3 min, c at 23 min in BDM and d in the presence of KB-R7943. (C) Difference between I–V curves obtained by subtraction of the currents in (B) as indicated.

The envelope of the current gradually decreased when 10 mM BDM was added to the external solution. The suppressing effect of BDM lasted for more than 10 min before a steady state was attained. Figure 1B illustrates the current-voltage (I–V) relations of the control (a), at 3 min (b) and at 23 min (c) in BDM. BDM-sensitive currents recorded at 3 and 23 min both crossed the control I–V curve at about a holding potential of −60 mV. After 23 min, 100 μM KB-R7943 was added to the BDM solution to completely block I_{NCX}. The I–V curve in the presence of KB-R7943 also crossed the control at −60 mV (Figure 1B(d)), confirming that the BDM-sensitive current was I_{NCX}. The net I–V curves of the BDM-sensitive components were obtained by subtracting those in KB-R7943 (d) from (a), (b) or (c) and they all crossed the voltage axis at −60 mV (Figure 1C).

Various concentrations of BDM were applied to obtain the concentration-inhibition curve (Figure 2). BDM inhibited I_{NCX} in a concentration-dependent manner. The current magnitude was measured at +50 mV for the outward component and at −100 mV for the inward component and the per cent inhibition was calculated by assuming that 100 μM KB-R7943 completely inhibited I_{NCX}. A sigmoid fitting of the curves yielded IC_{50} values for BDM of 2.4 and 2.3 mM and Hill coefficients of 1 and 0.9 for the outward and inward I_{NCX}, respectively. Thus, BDM inhibited both outward and inward I_{NCX} equally under these experimental conditions.

Effect of okadaic acid on the BDM inhibition of I_{NCX}

There is a report that BDM enhances the activity of phosphatases (Zimmermann *et al*., 1996). Therefore, we examined whether the inhibitory effect of BDM on I_{NCX} was due to the activation of phosphatases. Okadaic acid at nanomolar concentrations is known to inhibit phosphatases, especially proteinphosphatase 1 (PP1) and proteinphosphatase 2A (PP2A) (Cohen & Cohen, 1989). Figure 3 illustrates the effect of 1 μM okadaic acid in the pipette solution. In the

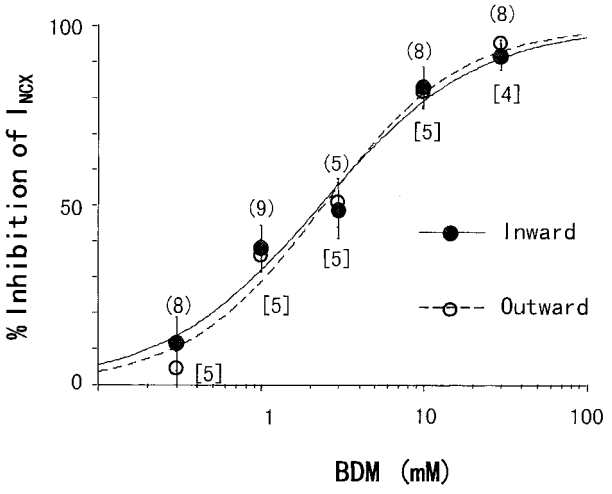

Figure 2 Concentration-inhibition curves of BDM. I_{NCX} was measured at +50 mV (outward) and −100 mV (inward component). The numbers of cells for outward (n) and inward [n] I_{NCX} components. The averages of the data points were fitted. IC_{50} of BDM were 2.4 and 2.3 mM and Hill coefficients 1 and 0.9 for the outward and inward I_{NCX}, respectively.

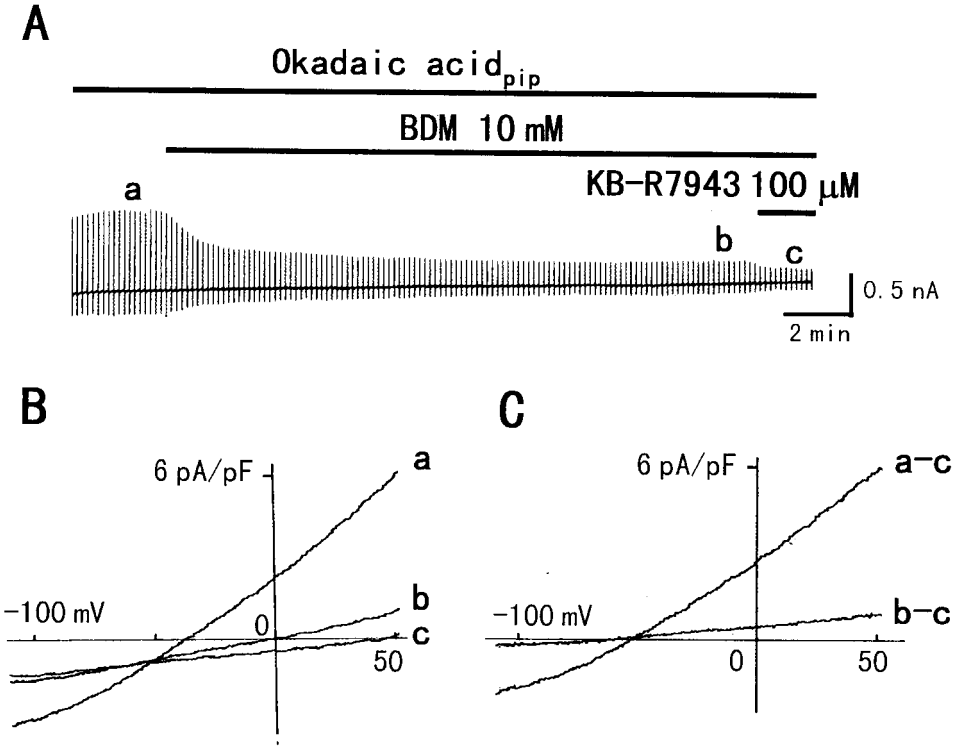

Figure 3 Effect of okadaic acid on the inhibition of I_{NCX} by BDM. (A) Current recording in the presence of 1 μM okadaic acid in the pipette solution. (B) I–V curves at the corresponding labels in (A). (C) Difference between I–V curves obtained from the corresponding labels in (B). Currents are expressed as current densities.

absence of okadaic acid, BDM inhibited I_{NCX} with a similar time-course to that illustrated in Figure 1. I–V curves obtained after about 20 min of BDM superfusion indicated that 10 mM BDM inhibited I_{NCX} at +50 mV by 81.5±3.9% ($n=5$) and 81.9±1.4% ($n=5$) in the absence and presence of okadaic acid, respectively. These values were not significantly different, indicating that okadaic acid-sensitive phosphatases are not involved in the inhibitory effect of BDM.

Effect of trypsin on the BDM inhibition of I_{NCX}

To see whether BDM inhibits I_{NCX} from inside or outside the cell membrane, we included trypsin (2.5 ~5 μg ml^{-1}) in the pipette solution. Cytoplasmic treatment with trypsin or α-chymotrypsin has been demonstrated to abolish various intracellular regulatory mechanisms on the Na^+/Ca^{2+} exchanger (Hilgemann, 1990; Doering & Lederer, 1993; Kimura, 1993). Figure 4 illustrates the effect of 10 mM BDM on I_{NCX} with trypsin in the pipette solution. The inhibitory effect of BDM disappeared in the presence of trypsin. Similar results were obtained in three other cells. The average KB-R7943 sensitive current density at +50 mV in the absence of trypsin was 6.8±0.4 pA/pF ($n=10$) while that in the presence of trypsin was 5.9±0.9 pA/pF ($n=4$). These values were not significantly different. The average BDM sensitive current component at +50 mV in the absence of trypsin was 5.3±0.4 pA/pF ($n=4$) which was approximately 78% of the control, while that in the presence of trypsin was 1.1±0.3 pA/pF ($n=4$) which was 18.6% of the control with trypsin. These data suggest that trypsin removed the intracellular site of action of BDM on the Na^+/Ca^{2+} exchanger. KB-R7943 at 100 μM completely blocked I_{NCX} even in the presence of trypsin, indicating that the site of action of KB-R7943 is not trypsin-sensitive.

Effect of PAM

BDM has been known as a chemical phosphatase (Holmstedt, 1959). If BDM inhibits I_{NCX} directly as a chemical phosphatase, it is possible that a similar oxime compound, PAM (praliodoxime) would also inhibit I_{NCX}. As shown in Figure 5, 10 mM PAM inhibited I_{NCX} by 74.7±1.5% ($n=4$) at +50 mV, which is similar to the effect of BDM (81.4±8.6%, $n=5$), though the onset of inhibition was slower than with BDM. This suggests that PAM and BDM might have inhibited I_{NCX} by a common mechanism.

Effects of isoprenaline and PMA on BDM-suppressed exchange current

If BDM inhibits I_{NCX} as a chemical phosphatase by dephosphorylating the exchanger, re-phosphorylation should

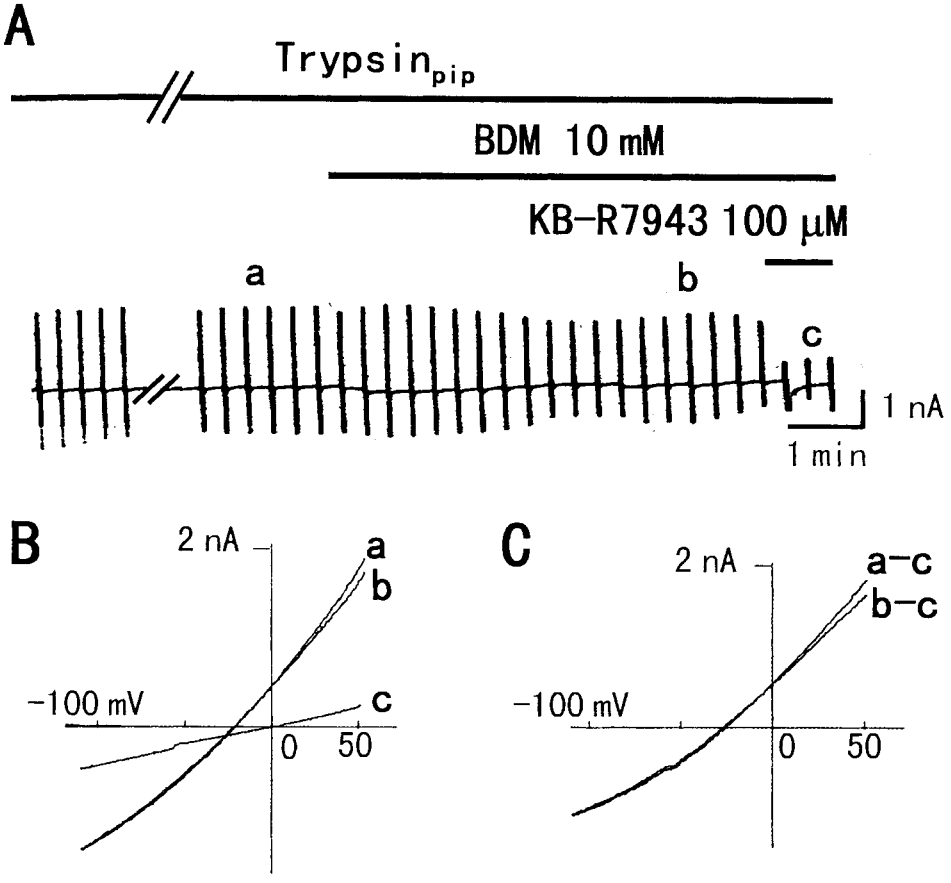

Figure 4 Effect of BDM on I_{NCX} in the presence of 2.5~5 μg ml^{-1} trypsin in the pipette solution. (A) Chart recording of the current. Horizontal bars above indicate the periods where trypsin, BDM and KB-R7943 were applied. BDM and KB-R7943 were perfused in the bath solution. (B) I–V curves obtained at the corresponding labels in (A). a is control, b in the presence of 10 mM BDM and c in the presence of BDM and 100 μM KB-R7943. (C) Difference of I–V curves between the currents illustrated in (B).

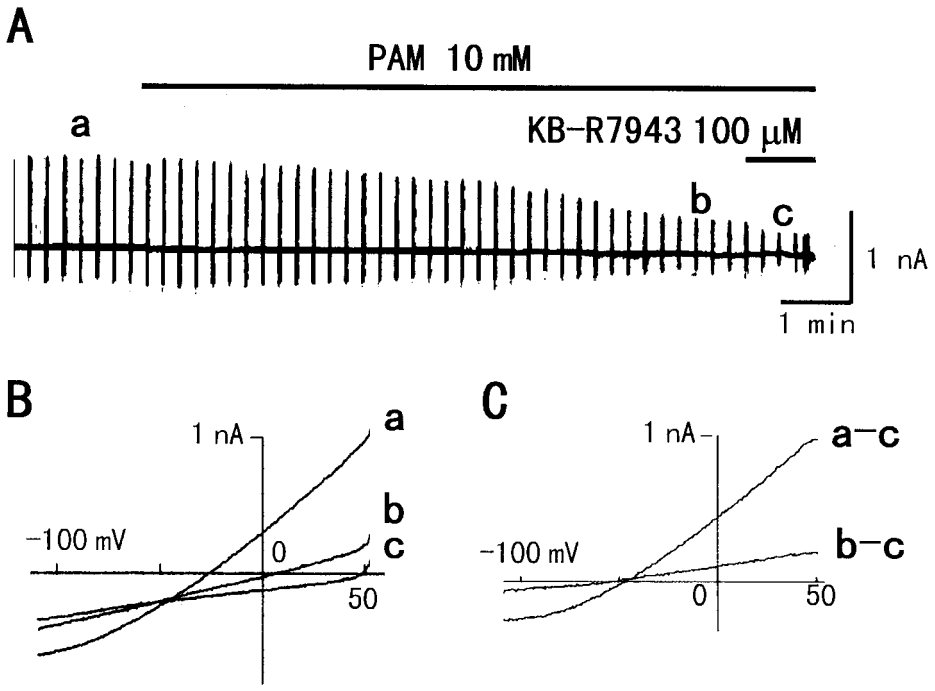

Figure 5 Effect of PAM on I_{NCX}. (A) Chart recording of the membrane current. The horizontal bars above indicate where 10 mM PAM and 100 μM KB-R7943 were applied externally. (B) I–V curves obtained at the corresponding labels in (A). a is control, b in the presence of 10 mM PAM and c in the presence of PAM and 100 μM KB-R7943. (C) Difference of I–V curves between a, b and c in (B) as indicated.

stop the inhibition by BDM and even restore I_{NCX}. The Na^+/Ca^{2+} exchanger is reported to have phosphorylation sites for protein kinase A, Ca^{2+}/calmodulin kinase II (Nicoll *et al*., 1990) and protein kinase C (Shigekawa *et al*., 1996). We tested 50 μM isoprenaline to stimulate the cyclic AMP-protein kinase A pathway *via* β-receptors in the presence of 5 mM DIDS (4,4′-dinitrostilbene-2,2′-disulphonic acid disodium salt), a cyclic AMP-induced Cl^- channel inhibitor, and also 8 μM PMA (phorbol 12-myristate 13-acetate) to activate protein kinase C. These did not stop the inhibitory effect of BDM or cause recovery of the inhibited I_{NCX} ($n=3$ for each, data not shown). Therefore, phosphorylation by PKA and PKC may not be involved in the effect of BDM.

Effects of BDM on ATP hydrolysis

We determined whether BDM dephosphorylates ATP and converts ATP to ADP and/or other metabolites. A mixture of 2.2 mM ATP and 10 mM BDM was allowed to stand for 5∼10 min, and then analysed by HPLC. No degradation of ATP was detected, indicating that BDM does not convert ATP to any other metabolites. Therefore, although BDM is called a chemical phosphatase, it does not dephosphorylate ATP.

Effect of BDM on expressed NCX1

The above results indicate that BDM inhibits I_{NCX} through a mechanism distinct from its chemical phosphatase activity. To clarify the mechanism of BDM inhibition of I_{NCX}, we used CCL39 cells stably expressing a dog heart NCX1 isoform and its mutant in which three serine residues in the internal loop were replaced with alanines (S249A/S250A/S357A). Therefore, this mutant does not have the major phosphorylation sites of the exchanger (Iwamoto *et al*., 1998). As shown in Figure 6A, 10 mM BDM decreased the I_{NCX} of the cells expressing the wild type NCX1 to $23.7\pm3.0\%$ ($n=5$) of the control value. The average current density of the wild type I_{NCX} was 5.0 ± 1.0 pApF^{-1} ($n=11$). However, BDM also suppressed the I_{NCX} of the mutated NCX1 (Figure 6B) to $23.6\pm3.5\%$ ($n=4$) of the control value, which was very similar to that of the wild type. The current density of the mutated I_{NCX} was 3.8 ± 2.3 pApF^{-1} ($n=4$), which was not significantly different than the value of the wild type (Figure 6D). The I-V curve of the untransfected CCL39 cells was insensitive to KB-R7943 (Figure 6C). Thus, BDM inhibited the exchanger which lacked phosphorylatable serine residues, strongly supporting the view that dephosphorylation is not involved in the inhibitory effect of BDM on the exchanger.

Discussion

In the present study, we demonstrated that in guinea-pig cardiac ventricular cells BDM inhibits both directions of I_{NCX} in a concentration-dependent manner with an IC_{50} of about 2.4 mM. The inhibition occurred at concentrations of BDM between 1 and 30 mM, which is similar to that for suppressing cardiac muscle contraction (Bergrey *et al*., 1981; West & Stephenson, 1989; Watanabe *et al*., 1996). The BDM IC_{50} value for suppressing contraction of guinea-pig papillary muscles was 1∼2 mM (Li *et al*., 1984). In the present study, intracellular trypsin treatment *via* the pipette solution

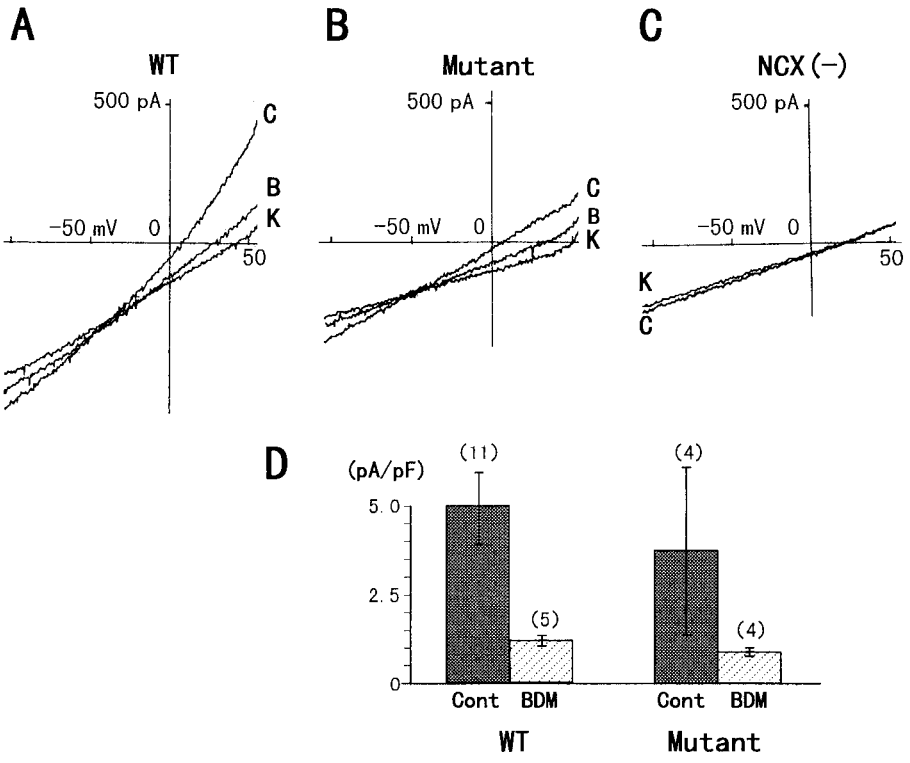

Figure 6 Effect of BDM on CCL39 cells transfected with wild type NCX1 (A), mutant with S249A/S250A/S357A (B), and cells without transfection (C). I–V curves were obtained by the same protocol used with cardiac myocytes. C, B and K indicate control, in the presence of 10 mM BDM and 100 μM KB-R7943, respectively. (D) Comparison of I_{NCX} densities measured at +50 mV with wild type and mutant NCX1 in the absence (Cont) and presence of 10 mM BDM (BDM).

prevented the inhibition of I_{NCX} by BDM. Similar results were also observed with amiodarone (Watanabe & Kimura, 2000). Chapman (1995) also reported the elimination of the inhibitory effect of BDM on L-type Ca channels in guinea-pig ventricular cells by trypsin treatment. Thus our result indicates that the site of action of BDM is on the cytoplasmic side of the cell membrane. Another oxime compound, PAM, also inhibited I_{NCX} in a similar manner to BDM. BDM and PAM might inhibit the exchanger by a common mechanism, possibly as 'chemical phosphatases', since both drugs were developed as dephosphorylating agents for organophosphate poisoning of acetylcholinesterase (Holmstedt, 1959). However, our other results do not support an inhibitory mechanism involving the chemical phosphatase action of BDM.

There are several potential PKA, PKC, and Ca^{2+}/CaM-dependent protein kinase II phosphorylation sites in the canine cardiac Na^+/Ca^{2+} exchanger (NCX1) (Nicoll *et al*., 1990; Iwamoto *et al*., 1996b; see review by DiPolo & Beaugé, 1999). Guinea-pig and dog heart Na^+/Ca^{2+} exchangers (NCX1) both have 970 amino acids with 98% homology (Tsuruya *et al*., 1994) and all the putative phosphorylation sites are conserved in the two species. Iwamoto *et al*. (1996b) demonstrated that dog heart NCX1 expressed in fibroblasts and NCX of rat neonatal cardiomyocytes exhibited a significant basal level of phosphorylation, which was enhanced by treatment with the PKC-activating agent, PMA. Furthermore, PKC activation by PMA caused phosphorylation of NCX1 and increased Na_+-dependent $^{45}Ca^{2+}$ uptake (Iwamoto *et al*., 1998).

In our hands, however, 0.8∼8 μM PMA or 50 μM isoprenaline did not reverse the BDM-induced inhibition of I_{NCX}. This result is different from the L-type Ca^{2+} current in dorsal root ganglia neurones, which BDM also inhibited but the inhibition was completely reversed by 8-bromo-cyclic AMP or 50 μM isoprenaline (Huang & McArdle, 1992). PMA or isoprenaline in the absence of BDM did not affect I_{NCX} in guinea-pig ventricular cells. We also confirmed by HPLC that BDM does not dephosphorylate ATP.

Zimmermann *et al*. (1996) reported that BDM enhanced the activity of phosphorylase phosphatases in guinea-pig ventricular homogenates. Protein phosphatses (PP) dephosphorylate protein serine/threonine residues specifically (reviewed by Herzig & Neumann, 2000). Okadaic acid inhibits PP1 and PP2A at nanomolar concentrations (Cohen & Cohen, 1989). Since the inhibitory effect of 10 mM BDM on I_{NCX} was not affected by 1 μM okadaic acid in the pipette solution (Figure 3), the inhibition was not due to the activation of PP1 or PP2A. In agreement with our results, it has been reported that not only the exchange activity was unaffected by various agents which alter protein phosphorylation, such as okadaic acid, calyculin, staurosporine and calphostine C, but also a phosphorylated form of the exchanger was not detected in Chinese hamster ovary cells and COS cells expressing the bovine cardiac Na^+/Ca^{2+} exchanger after incubation with [^{32}P]-phosphate (Condorescu *et al*., 1995).

Conclusive evidence was obtained by using CCL39 fibroblast cells transfected with the dog heart NCX1 and with its mutant lacking the three serine residues (at 249, 250

and 357) in the internal loop which were replaced with alanines, eliminating the major phosphorylation sites of the exchanger (Iwamoto *et al*., 1998). BDM inhibited both types of I_{NCX} with similar potency. This suggests that the phosphorylation sites are not necessary for inhibition by BDM and that BDM inhibits I_{NCX} by a mechanism other than dephosphorylation of the exchanger as a 'chemical phosphatase'. Similar results were obtained by Allen *et al*. (1998) who tested BDM on wild type and mutant Ca^{2+} channels expressed in *Xenopus* oocyes. The mutant Ca^{2+} channels, which lacked protein kinase A-dependent phosphorylation sites, were also inhibited by BDM with a potency equal to its inhibition of the wild type Ca^{2+} current.

Recently, a new regulatory effect of ATP *via* phosphatidylinositols on the mammalian cardiac Na^+/Ca^{2+} exchanger with the giant patch-clamp method was suggested (Hilgemann, 1997). Phosphatidyl inositol-4,5-bisphosphate (PIP_2) increased cardiac I_{NCX} (Hilgemann & Ball, 1996). ATP increases PIP_2 formation from phosphatidyl inositol (PI) by PI kinases. The exchanger may be directly regulated by PIP_2 through its binding to a positively charged cytoplasmic regulatory domain most likely the XIP region (Hilgemann & Ball, 1996; He *et al*., 2000). Therefore, we cannot exclude the possibility that BDM might affect the formation of PIP_2.

The initial aim of this study was to examine the possibility of using BDM as a tool for suppressing contraction of cardiac cells while investigating I_{NCX}. BDM suppressed I_{NCX} over a concentration range similar to that for suppressing contraction. Therefore, BDM cannot be considered an ideal tool for investigating I_{NCX}. Evidence that BDM affects ion channels, but not by a dephosphorylation mechanism has been accumulating with various cell types (recently reviewed by Herzig & Neumann, 2000). An alternative mechamism for the inhibition by BDM of various ion channels and a transporter like NCX is not known. More experiments are required to elucidate the precise mechanism of action of BDM.

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