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Volume-sensitive Chloride Channels Involved in Apoptotic Volume Decrease and Cell Death

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Abstract. Apoptosis is an essential process in organ development, tissue homeostasis, somatic cell turnover, and the pathogenesis of degenerative diseases. Apoptotic cell death occurs in response to a variety of stimuli in physiological and pathological circumstances. Efflux of K^+ and Cl^- leads to apoptotic volume decrease (AVD) of the cell. Both mitochondrion-mediated intrinsic, and death receptor-mediated extrinsic, apoptotic stimuli have been reported to rapidly activate Cl^- conductances in a large variety of cell types. In epithelial cells and cardiomyocytes, the AVD-inducing anion channel was recently determined to be the volume-sensitive outwardly rectifying (VSOR) Cl^- channel which is usually activated by swelling under non-apoptotic conditions. Blocking the VSOR Cl^- channel prevented cell death in not only epithelial and cardiac cells, but also other cell types, by inhibiting the induction of AVD and subsequent apoptotic events. Ischemia-reperfusion-induced apoptotic death in cardiomyocytes and brain neurons was also prevented by Cl^- channel blockers. Furthermore, cancer cell apoptosis induced by the anti-cancer drug cisplatin was recently found to be associated with augmented activity of the VSOR Cl^- channel and to be inhibited by a Cl^- channel blocker. The apoptosis-inducing VSOR Cl^- channel is distinct from ClC-3 and its molecular identity remains to be determined.

Key words: Anion channel — VSOR Cl^- channel — Apoptotic volume decrease — Apoptosis — Ischemia-reperfusion injury — Anti-cancer drug

Introduction

Anion channels play an electrogenic role in excitable cells, shifting the membrane potential, and a Cl^--transporting role in epithelial cells which secrete or absorb isotonic NaCl fluids. Recent studies have demonstrated more general roles for anion channels in animal cells. These include roles in cell volume regulation (Okada, 1997, 2004), cell proliferation or the cell cycle (Shen et al., 2000; Wondergem et al., 2001) and cell death (Gulbins et al., 2000; Okada et al., 2001, 2004). Of a number of different anion channels, the most important is the volume-sensitive outwardly rectifying (VSOR) Cl^- channel, a major type of swelling-activated anion channel phenotypically characterized by volume sensitivity (i.e., activation and inhibition by cell swelling and shrinkage, respectively), outward rectification, intermediate unitary conductance, low-field anion selectivity, broad-spectrum sensitivity to anion channel blockers and cytosolic ATP dependence (Okada, 1997). Here we summarize roles of the VSOR Cl^- channel in apoptosis.

Apoptotic Stimulation Activates VSOR Cl^- Channel Currents

Previous patch-clamp studies have shown activation of anion currents in a wide variety of cell types in response to a number of apoptotic stimuli, including the bacterial alkaloid staurosporine (Coca-Prados et al., 1995; Porcelli et al., 2003, 2004; Okada et al., 2004; Shimizu, Numata & Okada, 2004; Elinder et al., 2005), the death receptor ligand TNFα (Schumann, Gardner & Raffin, 1993; Nietsch et al.; 2000; Shimizu et al., 2004), Fas ligand (Szabo et al., 1998; Shimizu et al., 2004), doxorubicin (d'Anglemont de Tassigny et al., 2004), C_2-ceramide (d'Anglemont de Tassigny et al., 2004), sphingolipids (Souktani et al., 2000), lipopolysaccharide (Meng, Carruth & Weinman, 1997), *Pseudomonas aeruginosa* (Ullrich et al., 2003) and H_2O_2 (Browe & Baumgarten 2004; Shimizu et al., 2004; Varela et al., 2004).

Many of the anion currents studied in relation to apoptotic cell death resemble VSOR Cl^- channel currents in outward rectification (Souktani et al.,

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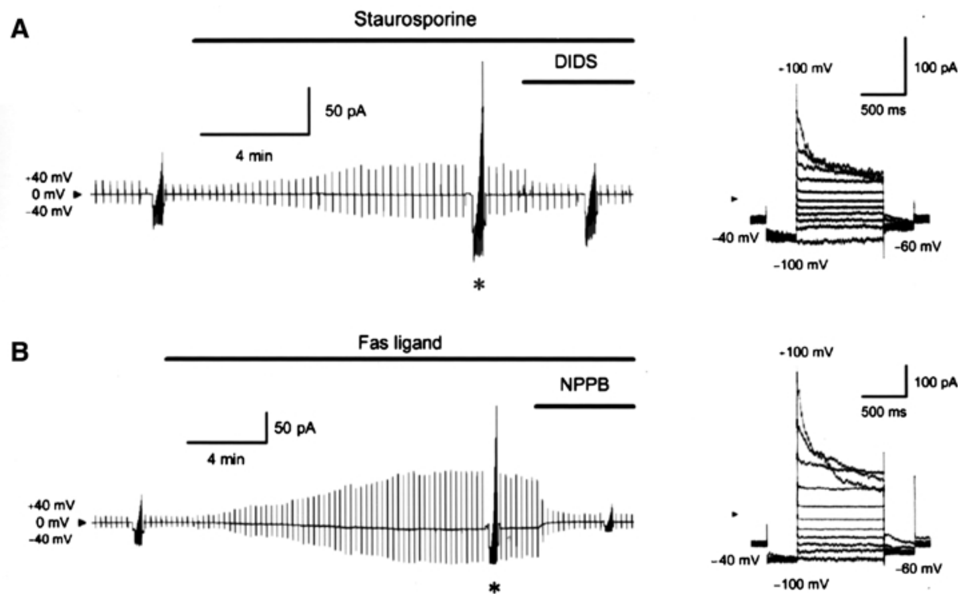

Fig. 1. Activation of whole-cell VSOR Cl^- currents by a mitochondrion-mediated apoptosis inducer, staurosporine (*A*), and by a receptor-mediated apoptosis inducer, anti-Fas antibody (*B*), in HeLa cells. *Left panels*: Representative records of currents elicited by application of alternating pulses from 0 to ± 40 mV before and during exposure to staurosporine (4 μM), anti-Fas antibody (500 ng/ml), DIDS (500 μM) or NPPB (100 μM). *Right panels*: Expanded traces of current responses to step pulses from −100 to + 100 mV in 20-mV increments applied at asterisks in left-panel records. Experimental conditions were the same as those described previously (Shimizu et al., 2004).

2000; d'Anglemont de Tassigny et al., 2004; Porcelli et al., 2004; Shimizu et al., 2004), cytosolic ATP dependence (Nietsch et al., 2000; Shimizu et al., 2004), and sensitivity to NPPB (Nietsch et al., 2000; Souktani et al., 2000; Porcelli et al., 2004; Shimizu et al., 2004) and to phloretin (Porcelli et al., 2004). However, volume sensitivity has never been tested for the VSOR-like Cl^- currents recorded in *Xenopus* oocytes (Souktani et al., 2000), human hepatoma HTC cells (Nietsch et al., 2000), human endothelial ECV304 cells (Porcelli et al., 2004) and rabbit ventricular myocytes (d'Anglemont de Tassigny et al., 2004). An involvement of the VSOR Cl^- channel in AVD was suggested by the following results obtained in human epithelial HeLa, lymphoid U937 and rodent neuronal PC12 and NG108-15 cells (Maeno et al., 2000): First, cells undergoing AVD exhibit facilitated regulatory volume decrease (RVD), which involves VSOR Cl^- channel activity (Okada, 1997); second, AVD is abolished by glibenclamide, which blocks both CFTR and VSOR Cl^- channels (Liu et al., 1998); and third, AVD is also inhibited by phloretin, which blocks the VSOR Cl^- channel at low concentrations, but neither the cAMP-activated CFTR Cl^- channel nor the Ca^{2+}-activated Cl^- channel (Fan et al., 2001). Our recent study (Shimizu et al., 2004) has provided clear evidence that intrinsic (staurosporine) and extrinsic (Fas ligand or TNFα) inducers of apoptosis can both activate the VSOR Cl^- channel in human epithelial HeLa cells. The currents were sensitive to hypertonicity and cytosolic ATP removal as well as to DIDS and NPPB, as shown in Fig. 1. Here, in Fig. 2*A*, we show staurosporine-induced activation of VSOR Cl^- current in mouse ventricular myocytes in primary culture. Apoptosis induced by the anti-cancer drug cisplatin has also been shown to be associated with augmented activity of the VSOR Cl^- channel in cancer KB cells (Ise et al., 2006).

Although activation of the VSOR Cl^- channel is induced by cell swelling, activation of the channel takes place during the AVD process in non-swollen or even shrunken apoptotic cells (*see* Fig. 2*B* and Fig. 3). Since this apoptosis-associated activity of the VSOR Cl^- channel subsides upon more drastic cell shrinkage induced by hypertonicity (Shimizu et al., 2004), the threshold cell volume at which channel opening is triggered, termed the channel volume set point (Cannon, Basavappa & Strange, 1998), must be shifted to a lower level in apoptotic cells. The exact mechanism for a reduced set point is not known. However, reactive oxygen species (ROS) are known to be involved in activation of the VSOR Cl^- channel that takes place upon induction of apoptosis by staurosporine (but not Fas ligand or TNFα) in HeLa cells (Shimizu et al., 2004). A Src tyrosine kinase, p56Lck, was reported to mediate activation of VSOR-like Cl^- currents in lymphocytes stimulated with Fas ligand (Szabo et al., 1998) and by cell swelling (Lepple-Wienhues et al., 1998). It is also possible that the shift of the channel volume set point is related to elevation of the cytosolic ATP level, which has recently been found to occur in apoptotic

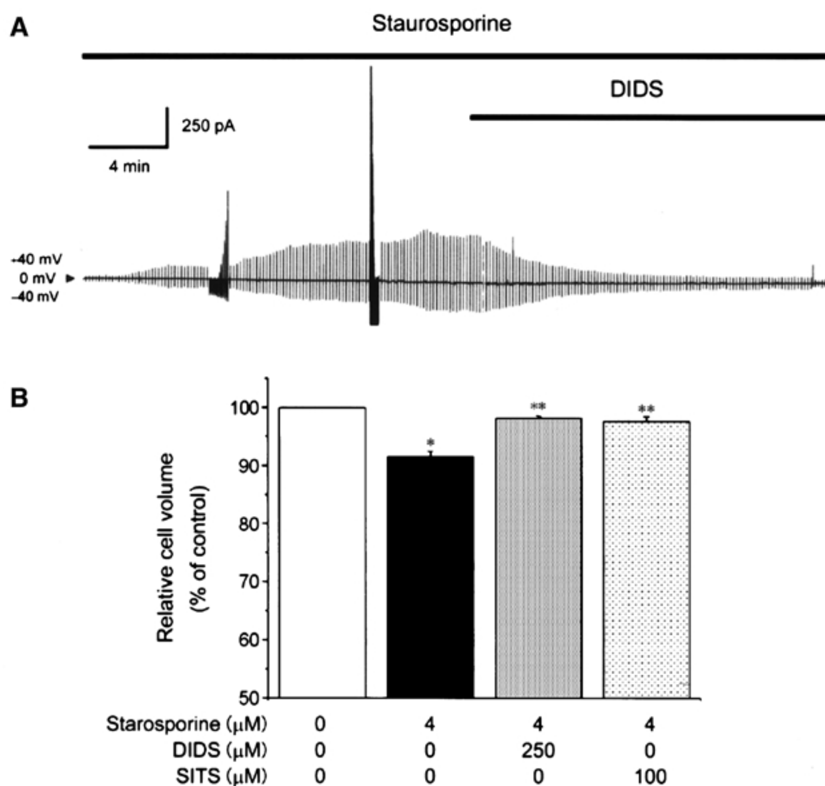

Fig. 2. Staurosporine-induced activation of VSOR Cl^- currents (*A*) and induction of AVD (*B*) in mouse ventricular cardiomyocytes in primary culture. (*A*) Representative record of whole-cell current responses to alternating pulses from 0 to ± 40 mV before and during application of staurosporine (4 μM) or DIDS (250 μM). Experimental conditions were the same as those described previously (Gong et al., 2004). (*B*) Relative cell volume before and 30 min after exposure to staurosporine in the absence or presence of DIDS or SITS. Cell volume measurements were performed by an electronic cell-sizing technique, as reported previously (Maeno et al., 2000). The data represent the mean ± SEM (vertical bars) of 9–12 experiments. * and **, significantly different ($P < 0.05$) from the control data (treated with vehicle, DMSO, alone) and from the staurosporine data in which stilbene-derivative Cl^- channel blockers were absent, respectively.

cells (Zamaraeva et al., 2005), because activity of the VSOR Cl^- channel is known to be dependent on intracellular ATP (Jackson, Morrison & Strange, 1994; Oiki, Kubo & Okada, 1994; Jackson et al., 1996; Meyer & Korbmacher, 1996; Okada, 1997).

AVD Induction Is Prevented by VSOR Cl^- Channel Blockers

A major hallmark of apoptosis is normotonic cell shrinkage, known as AVD, which starts before cell fragmentation into apoptotic bodies (Maeno et al., 2000; Okada & Maeno, 2001; Okada et al., 2001; Bortner & Cidlowski, 2002). Here we present, for the first time, data showing that AVD occurs in mouse cardiomyocytes treated with staurosporine (Fig. 2*B*, *second column*). DIDS and SITS, stilbene-derivative Cl^- channel blockers, eliminated AVD induction in mouse cardiomyocytes treated with staurosporine (Fig. 2*B*: *third* and *fourth columns*). In neuronal NG108-15 cells, AVD was induced by staurosporine (STS) or TNFα in a time-dependent manner, but was never observed in the presence of a non-specific Cl^- channel blocker (DIDS or NPPB), a VSOR Cl^- channel blocker (phloretin) or a K^+ channel blocker (quinine or Ba^{2+}), as shown in Fig. 3 (*A* and *B*: *top panels*). These Cl^- channel blockers were found to prevent staurosporine- or TNFα-induced AVD not only in NG108-15 cells but also in epithelial HeLa, lymphoid U937 and neuronal PC12 cells (Maeno et al., 2000); in addition, they prevented Fas ligand-induced AVD in U937 cells (Okada et al., 2001). A similar sensitivity of AVD to Cl^- channel blockers was observed in *Xenopus* oocytes with NPPB (Souktani et al., 2000), in rabbit ventricular myocytes with NPPB and IAA-94 (d'Anglemont de Tassigny et al., 2004), in human erythrocytes with NPPB and niflumic acid (Myssina et al., 2004), in mouse cortical neurons with DIDS, SITS and NPPB (Wei et al., 2004) and in human endothelial ECV304 cells with phloretin (Porcelli et al., 2004).

AVD induction was found to precede cytochrome *c* release, caspase-3 activation, DNA laddering and morphological changes seen under thin section electron microscopy in epithelial, lymphoid and neuronal cells after apoptotic stimulation with intrinsic and extrinsic inducers of apoptosis (Maeno et al., 2000). Figure 3 shows representative data obtained in NG108-15 cells. AVD induction (*top panels*) started earlier than caspase-3 activation (*middle panels*) and cell death induction (*bottom panels*). In addition, broad-spectrum caspase blockers failed to prevent staurosporine-induced AVD (Maeno et al., 2000; Okada & Maeno, 2001). Furthermore, cells were rescued from apoptotic cell death when the induction of AVD was eliminated by application of Cl^- or K^+ channel blockers (Maeno et al., 2000) or of a ROS scavenger, which caused the inhibition of VSOR Cl^- channels acti-

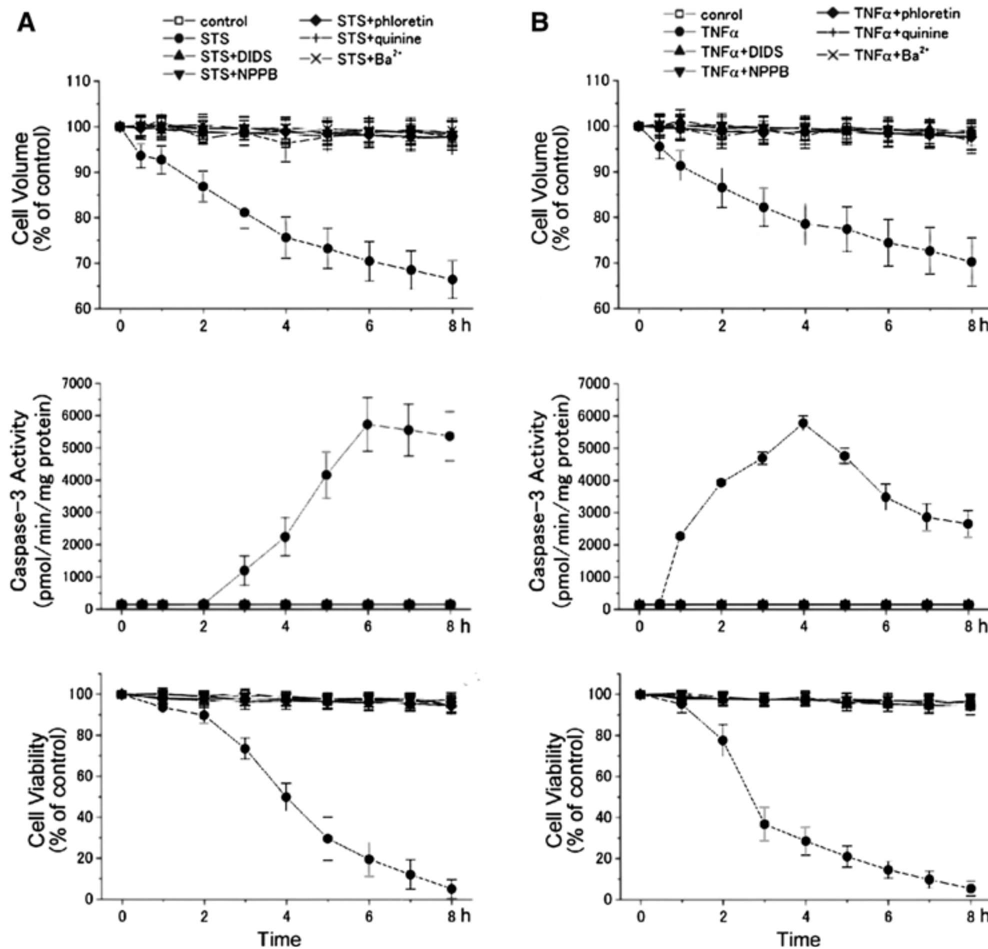

Fig. 3. The time course of AVD induction (*top panels*), caspase-3 activation (*middle panels*) and cell death (*bottom panels*) in NG108-15 cells induced by treatment with staurosporine (STS: 4 μM) (*A*) or TNFα (2 ng/ml *plus* cycloheximide 0.1 μg/ml) (*B*), and their prevention by simultaneous treatment with channel blockers, (500 μM DIDS, 500 μM NPPB, 30 μM phloretin, 500 μM quinine or 5 mM Ba^{2+}). Each date point represents the mean ± SEM (vertical bar) of 16 experiments. Experimental techniques employed were described in a previous paper (Maeno et al., 2000).

vated by staurosporine (Shimizu et al., 2004). Finally, it is known that hypertonicity-induced cell shrinkage per se results in activation of the apoptotic process in cells that lack a regulatory volume increase (RVI) response (Bortner & Cidlowski, 1996). The above results led us to conclude that AVD is an early prerequisite to apoptotic events leading to cell death (Maeno et al., 2000; Okada & Maeno, 2001; Okada et al., 2001). However, this concept has been challenged by several recent reports. First, Hortelano et al. (2002) observed that the VSOR Cl^- channel blockers phloretin and SITS blocked AVD induced by exposure to NO without preventing apoptotic death in RAW264.7 macrophages. Judging from the smear-like DNA degradation (shown in Fig. 4*B* in that paper) and minimal activation of caspase-3 (*see* Figs. 4*D* and 5*C* for RAW264.7 in comparison to Figs. 2*C* and 3*D* for Jurkat and HeLa in that paper), however, it might be possible that the cell death observed in NO-treated RAW264.7 cells was necrotic, not apoptotic, cell death. Second, Bortner & Cidlowski (2003) reported that 6 h after treatment with Fas ligand, Jurkat T cells exhibit swelling in conjunction with a number of apoptotic characteristics under low Na^+ conditions. However, it must be stressed that AVD represents early phase shrinkage of apoptotic cells and must be examined before cells undergo fragmentation (usually within 2 h after apoptotic stimulation: *see* Maeno et al., 2000) and before secondary necrosis starts. Third, Wei et al. (2004) observed in cortical neurons that Cl^- channel blockers (DIDS, SITS and NPPB) eliminated AVD but only partially inhibited cell death monitored by LDH release 9 to 15 h after treatment with staurosporine or ceramide. K^+ channel blockers (TEA and clofilium), on the other hand, eliminated both completely. However, it must be noted that in principle, apoptosis-associated anion efflux must have been mediated by an electroconductive pathway, if apoptosis-inducing K^+ efflux was mediated by K^+ channels. Also, the following practical points should be made: In that study, reduction of cell viability was monitored by LDH release, which may not represent apoptotic cell death alone, and the effects of Cl^- channel blockers (in contrast to those of K^+ channel blockers) were examined using concentrations that were too low.

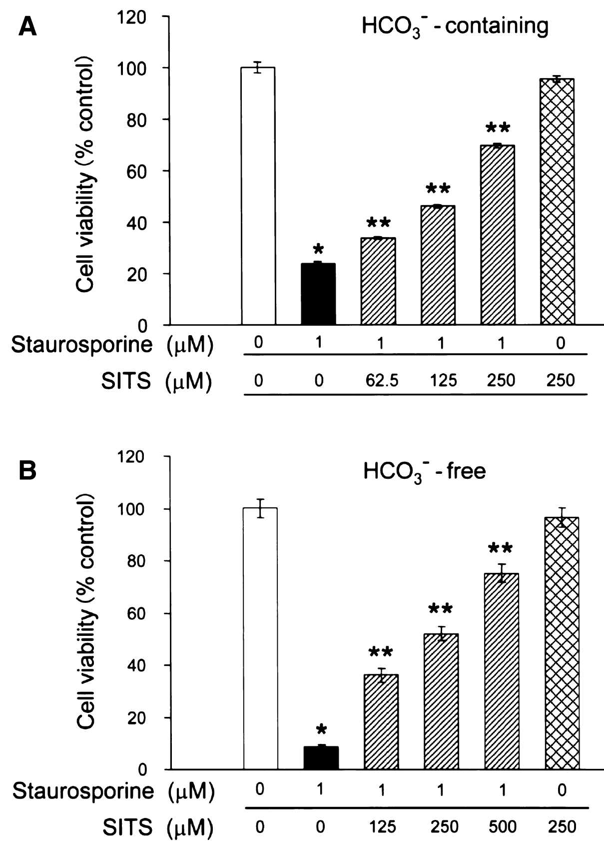

Fig. 4. Staurosporine-induced cell death and its prevention by SITS in rat ventricular cardiomyocytes in primary culture, with ambient HCO_3^- present (*A*) or absent (*B*). Cell viability was monitored by the MTT assay 18 h after a 2 h treatment with staurosporine ± SITS, as reported previously (Tanabe et al., 2005). In the control experiments (*white columns*), vehicle (DMSO) alone was applied. Each column represents the mean ± SEM (vertical bar) of 8 observations. * and **, significantly different from the control data and the data with staurosporine, respectively.

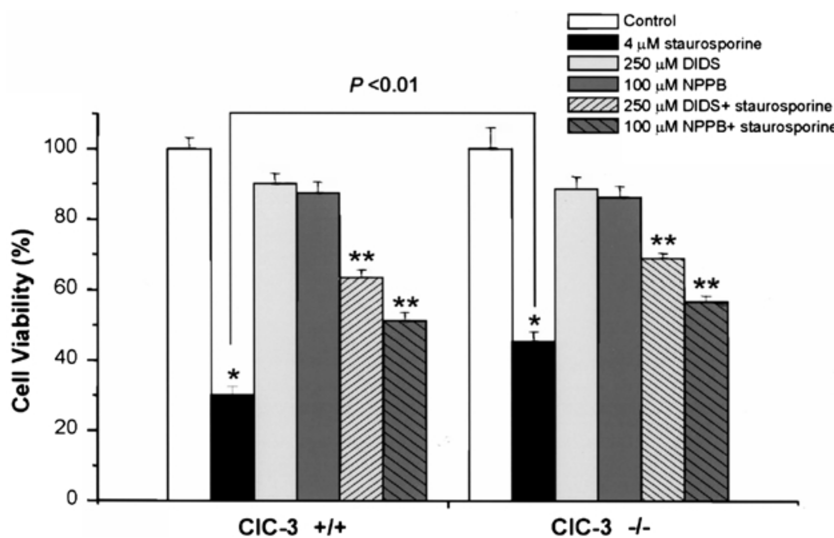

Fig. 5. Staurosporine-induced cell death and its prevention by DIDS or NPPB in ventricular cardiomyocytes isolated from wild-type mice and ClC-3 null-mutant mice. Each column represents the mean ± SEM (vertical bar) of 6 experiments. * and **, significantly different from the control data (treated with the vehicle, DMSO, alone) and the data with staurosporine alone, respectively. Cell viability was monitored by MTT assay, as described previously (Wang et al., 2005), 4 h after a 4-h treatment with staurosporine. Note that staurosporine-induced cell death in ClC-3-deficient (ClC-3 −/−) cardiomyocytes is significantly less prominent than that in wild-type (ClC-3 +/+) cardiomyocytes.

Apoptotic Cell Death Is Inhibited by Blocking of VSOR Cl⁻ Channels

To date, it has been reported that apoptotic cell death induced by chemicals conventionally employed as apoptosis inducers in a wide variety of cell types is prevented by anion channel blockers that are effective in inhibiting VSOR Cl⁻ channels. These blockers include DIDS (Fujita, Yanagisawa & Ishikawa, 1997; Fujita et al., 1999; Maeno et al., 2000; Barriere

et al., 2001; Araki et al., 2002; O'Reilly et al., 2002; Takahashi et al., 2005; Tanabe et al., 2005; Wang et al., 2005; Ise et al., 2006), SITS (Maeno et al., 2000; O'Reilly et al., 2002; Small, Tauskela & Xia, 2002), NPPB (Rasola et al., 1999; Maeno et al., 2000; Nietsch et al., 2000; d'Anglemont de Tassigny et al., 2004; Myssina et al., 2004; Takahashi et al., 2005; Tanabe et al., 2005; Wang et al., 2005), DPC (Nietsch, 2000), IAA-94 (Szabo et al., 1998; d'Anglemont de Tassigny et al., 2004), niflumic acid (Maeno et al., 2000; Kim, Kang & Lee, 2003; Myssina et al., 2004), flufenamic acid (Kim et al., 2003), glibenclamide (Szabo et al., 1998; Maeno et al., 2000; Barriere et al., 2001), phloretin (Maeno et al., 2000; Porcelli et al., 2004; Wang et al., 2005) and ROS scavengers (Shimizu et al., 2004; Wang et al., 2005). Recently, Cl^- channel blockers were also found to inhibit apoptotic death in cancer cells treated with the anti-cancer drug cisplatin (Ise et al., 2006), in cardiomyocytes subjected to ischemia-reperfusion in vitro (Wang et al., 2005), and in hippocampal neurons subjected to ischemia-reperfusion in vivo (Inoue et al., 2005).

As shown in Fig. 3 (*bottom panels*), cell viability monitored by mitochondrial dehydrogenase activity was sustained even 8 h after exposure to Cl^- channel blockers (Maeno et al., 2000). In assays of cell viability by trypan blue exclusion, it was found that these Cl^- channel blockers never caused non-specific damage in epithelial, lymphoid or neuronal cells stimulated with staurosporine or TNFα (Maeno et al., 2000). Thus, it is unlikely that these Cl^- channel blockers caused the cells to switch from apoptotic to necrotic cell death.

It was reported that the Cl^-/HCO_3^- exchanger is involved in the mechanism of apoptosis induction in bovine endothelial cells (Fujita et al., 1997, 1999), rat cardiomyocytes (Fujita et al., 1999) and human HeLa cells (Dong, Wang & Zhong, 2003). Since the stilbene derivatives DIDS and SITS are known blockers of both Cl^- channels and Cl^-/HCO_3^- exchangers, it is possible that the main target of stilbene derivatives is the anion exchanger. Recently, we have ruled out this possibility by observing effects of the removal of ambient HCO_3^-, which minimizes the operation of Cl^-/HCO_3^- exchangers. First, the inhibitory effect of DIDS on staurosporine-induced apoptosis was not affected by HCO_3^- removal in ventricular myocytes isolated from hearts of rat (Tanabe et al., 2005) and mouse (Takahashi et al., 2005). Second, the ability of DIDS to rescue mouse cardiomyocytes from ischemia-reperfusion-induced apoptosis was the same whether or not HCO_3^- was present (Wang et al., 2005). Third, we present evidence here that SITS also inhibits apoptosis in rat cardiomyocytes treated with staurosporine, even in the absence of ambient HCO_3^- (Fig. 4).

Molecular Identity of Anion Channel Involved in Apoptotic Cell Death Is Matter of Debate

The VSOR Cl^- channel is ubiquitously expressed in animal cells (Strange, Emma & Jackson, 1996; Nilius et al., 1997; Okada, 1997). Nevertheless, there is still a paucity of molecular information on this channel. The candidate protein most recently proposed was ClC-3 (Duan et al., 1997). Recent studies with ClC-3 knockout mice, however, provided clear evidence that functional expression of the VSOR Cl^- channel is independent of the molecular expression of ClC-3 in mouse hepatocytes and pancreatic acinar cells (Stobrawa et al., 2001), in mouse parotid salivary acinar cells (Arreola et al., 2002) and in mouse ventricular cardiomyocytes (Gong et al., 2004). Meanwhile, increased tyrosine phosphorylation of ClC-3 has been observed in association with apoptotic stimulation with tributyltin in T lymphoma cells (Dupéré-Minier et al., 2004). Thus, this result boosts the hypothesis that VSOR-like Cl^- channel currents activated by an apoptosis inducer are mediated by ClC-3. However, our recent studies have demonstrated that staurosporine-induced caspase-3 activation, DNA laddering and cell death (Fig. 5) in ClC-3 null-mutant mouse cardiomyocytes, and their inhibition by DIDS and NPPB are essentially the same as in wild-type mouse cardiomyocytes (Wang et al., 2005). Thus, it is clear that the ClC-3 protein is distinct from the protein constituting the pore of the VSOR Cl^- channel involved in apoptosis. As shown in Fig. 5, however, reduction of cell viability was slightly but significantly less prominent in ClC-3-deficient cardiomyocytes, suggesting a possible regulatory role of ClC-3 in the apoptotic process.

Elinder et al. (2005) have recently observed increased activity of maxi-anion channels in association with staurosporine-induced apoptosis in mouse neuronal HT22 cells that constitutively express plasmalemmal VDAC (pl-VDAC) proteins. Since pl-VDAC has long been assumed to be the molecule corresponding to the maxi-anion channel, which exhibits a current profile similar to that of mitochondrial VDAC (Dermietzel et al., 1994; Buettner et al., 2000; Steinacker et al. 2000), it was suggested that pl-VDAC is responsible for the maxi-anion channel activity associated with neuronal apoptosis (Elinder et al., 2005). However, our preliminary study (Dutta & Okada, unpublished) using staurosporine-treated rat cardiomyocytes, which exhibit brisk activity of the volume-activated maxi-anion channel under hypotonic, hypoxic or ischemic conditions (Dutta et al., 2004), failed to detect increased activity of the channel. Furthermore, Sabirov et al. (2006) have recently provided compelling evidence that activity of the maxi-anion channel is completely independent of the expression of pl-VDAC proteins. There remains,

however, the possibility that increased NADH reductase activity, which is intrinsic to the pl-VDAC protein, is involved in staurosporine-induced apoptosis in HT22 cells (Elinder et al., 2005).

Previous studies suggested that in some cell types the apoptotic process involves other anion channels that are volume-independent, including the cAMP-activated Cl^- channel, CFTR (Gottlieb & Dosanjh, 1996; Barriere et al., 2001; Cannon et al., 2003), and the Ca^{2+}-activated Cl^- channel candidate, CLCA2 (Elble & Pauli, 2001). However, no direct evidence for an actual involvement of CFTR in apoptosis has as yet been provided. Also, it must be pointed out that recent studies argue against the possibility that CLCA proteins are the molecules corresponding to Ca^{2+}-activated Cl^- channels (*see* Eggermont, 2004). Thus, further investigation is needed before accurate identification of the molecule of the anion channel involved in apoptotic cell death.

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