

## Full-length article

# Interaction between Cl<sup>-</sup> channels and CRAC-related Ca<sup>2+</sup> signaling during T lymphocyte activation and proliferation<sup>1</sup>

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## Key words

T lymphocytes; lymphocyte activation; chloride channels; calcium; ion channels; interleukin-2;

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## Abstract

**Aim:** To test the hypothesis that Cl<sup>-</sup> channel blockers affect T cell proliferation through Ca<sup>2+</sup>-release-activated Ca<sup>2+</sup> (CRAC) signaling and examine the effects of the combination of a CRAC channel blocker and a Cl<sup>-</sup> channel blocker on concanavalin A (ConA; 5 mg/mL)-induced Ca<sup>2+</sup> signaling, gene expression and cellular proliferation in human peripheral T lymphocytes. **Methods:** [<sup>3</sup>H]Thymidine incorporation, Fura-2 fluorescent probe, RNase protection assay, and reverse transcription-polymerase chain reaction were used. **Results:** The Cl<sup>-</sup> channel blocker 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) inhibited ConA-induced Ca<sup>2+</sup> influx, interleukin-2 mRNA expression and T lymphocyte proliferation in a concentration-dependent manner, and also enhanced the inhibitory effects of 1- $\beta$ -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl-1H-imidazole (SK&F96365) on the above key events during T cell activation. A combination of DIDS (1  $\mu$ mol/L) and SK&F96365 (1  $\mu$ mol/L) significantly diminished ConA-induced CIC-3 mRNA expression by 64%, whereas DIDS (1  $\mu$ mol/L) or SK&F96365 (1  $\mu$ mol/L) alone decreased ConA-induced CIC-3 mRNA expression by only 16% and 9%, respectively. **Conclusion:** These results suggest that there is an interaction between CRAC-mediated Ca<sup>2+</sup> signaling and DIDS-sensitive Cl<sup>-</sup> channels during ConA-induced T cell activation and proliferation. Moreover, the DIDS-sensitive Cl<sup>-</sup> channels may be related to the CIC-3 Cl<sup>-</sup> channels.

## Introduction

Activation of T lymphocytes with mitogen results in a biphasic elevation of intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>) due to Ca<sup>2+</sup> release from internal stores followed by a sustained Ca<sup>2+</sup> influx across the plasma membrane<sup>[1,2]</sup>. The latter phase is critical for subsequent calcineurin activation and interleukin (IL)-2 gene expression in activated T cells<sup>[3,4]</sup>. The voltage-independent calcium release activated Ca<sup>2+</sup> (CRAC) channels have been strongly suggested to be solely responsible for sustained Ca<sup>2+</sup> influx during mitogenic stimulation of T cells by lectins or antibodies<sup>[2-4]</sup>. The evidence indicating that concanavalin (ConA) induces Ca<sup>2+</sup> influx through CRAC channels includes: (1) the Ca<sup>2+</sup> currents induced by mitogen stimulation and by passive store depletion [using intracellular ethyleneglycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) or an inhibitor of endoplasmic reticu-

lum Ca<sup>2+</sup>-ATPases that deplete internal Ca<sup>2+</sup> stores and irreversibly activate store-operated Ca<sup>2+</sup> (CRAC) channels in the plasma membrane] share the same properties, including ion selectivity, conductance, Ca<sup>2+</sup>-dependent inactivation, and blockage by heavy metals (Ni<sup>2+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup>, Cd<sup>2+</sup>)<sup>[3,5]</sup>; (2) stimulation of T cells with lectins or antibodies does not further increase Ca<sup>2+</sup> influx or Ca<sup>2+</sup> current in T cells whose stores are already depleted by thapsigargin (TG)<sup>[3,6]</sup>; (3) 1- $\beta$ -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl-1H-imidazole (SK&F96365), a CRAC channel blocker, blocks  $I_{CRAC}$  in parallel with the [Ca<sup>2+</sup>]<sub>i</sub> rise and induction of T cell activation markers by TG<sup>[4]</sup>; and (4) a specific defect in Ca<sup>2+</sup> influx associated with the absence of  $I_{CRAC}$  was shown in nonresponsive T cells from patients with a severe immunodeficiency<sup>[7]</sup>, and, likewise, T cell activation with anti-CD3 evokes Ca<sup>2+</sup> release but not influx in Jurkat mutants selected specifically for the absence of store-oper-

ated  $\text{Ca}^{2+}$  entry<sup>[8]</sup>.

$\text{Cl}^-$  channels are involved in the proliferation of a variety of cell types, such as endothelial cells, glioma cells, hepatocytes, and vascular smooth muscle cells<sup>[9-14]</sup>. Previous studies (including our own) have also shown that T cell activation and proliferation were inhibited by  $\text{Cl}^-$  channel blockers [4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), 5-nitro-2-(5-phenylpropylamino)-benzoate (NPPB) and tamoxifen] as well as by antisense oligonucleotides against  $\text{ClC-3}$ <sup>[15-19]</sup>. We hypothesized that these  $\text{Cl}^-$  channel blockers affected T cell proliferation through  $\text{Ca}^{2+}$ -signaling and the interaction between  $\text{Cl}^-$  channels and CRAC channels that was involved in T lymphocyte activation and proliferation.

The present work was designed to further examine several key events in ConA-induced T cell activation and proliferation, including  $\text{Ca}^{2+}$  signaling, IL-2 mRNA expression, and final T cell proliferation. The functional role of  $\text{Cl}^-$  and CRAC channels in T cell activation was confirmed by a [<sup>3</sup>H]thymidine proliferation assay using several kinds of channel blockers. To investigate whether there is an interaction between  $\text{Cl}^-$  and CRAC channels in stimulated T cells, we further compared the effects of a combination of these channel blockers and each alone on ConA-induced  $\text{Ca}^{2+}$  signaling, IL-2 mRNA expression, and final T cell proliferation by using [<sup>3</sup>H]thymidine incorporation, Fura-2 analysis of  $\text{Ca}^{2+}$  movement, RNase protection assay (RPA) of human IL-2 mRNA expression and reverse transcription-polymerase chain reaction (RT-PCR) analysis of  $\text{ClC-3}$  mRNA expression. Our data suggest an interplay between DIDS-sensitive  $\text{Cl}^-$  channels and CRAC-related  $\text{Ca}^{2+}$  signaling during T cell activation and proliferation.

## Materials and methods

**Isolation of human T lymphocytes** Human venous blood was obtained from the Guangzhou Blood Center and T lymphocytes were isolated as described previously<sup>[20]</sup>. Briefly, venous blood from healthy adult donors was collected in heparinized tubes and diluted with RPMI-1640 containing 25 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10% heated-activated fetal calf serum (FCS), 2 mmol/L glutamine, 50 mg/mL streptomycin, and 50 U/mL penicillin. Peripheral blood mononuclear cells were centrifuged on Ficoll-Paque (Shanghai Reagent Institute, Shanghai, China), and then transferred to a sterile nylon wool fiber column (Polysciences Inc, Warrington, USA) pre-equilibrated with RPMI-1640/10% FCS at 37 °C for 45 min. The column was eluted with pre-warmed RPMI-1640/10% FCS, and the cells were washed 3 times with this medium. Cell populations

obtained in this way were  $\geq 95\%$  T lymphocytes, which were measured by fluorescence-activated cell sorter analysis with anti-CD3 antibody OKT3 (Ortho Pharmaceuticals, Raritan, NY, USA).

**Proliferation assay** Fresh isolated T lymphocytes were suspended in RPMI-1640/FCS and  $2 \times 10^5$  cells were placed in each well of a 96-well plate. The cells were preincubated with the compounds tested for 15 min and then exposed to mitogenic lectin and ConA (5  $\mu\text{g}/\text{mL}$ ). At a concentration of 5  $\mu\text{g}/\text{mL}$ , ConA induces significant proliferation of isolated human T lymphocytes without any toxic effects, which has been confirmed in many previous studies<sup>[1-3,15,16,18,21-23]</sup>. The cultures were maintained in a humidified incubator at 37 °C (in 5%  $\text{CO}_2$ ) for 72 h. Sixteen hours before the cells were harvested,  $18.5 \times 10^3$  Bq [<sup>3</sup>H]thymidine (Shanghai Atomic Energy Research Institute, Shanghai, China) was added to each well. The incorporated radioactivity was measured in a Beckman scintillation counter (Beckman, USA), expressed as counts per minute (3 replicates/experiment). In order to exclude the direct cytotoxic effects of the tested compounds on T cells, the trypan blue exclusion assay was used to determine cell viability at the end of incubation, and only samples with a viability of at least 95% were used for further assay. At the concentration used, no cytotoxic effect on cell cultures was found.

**[ $\text{Ca}^{2+}$ ]<sub>i</sub> measurements** [ $\text{Ca}^{2+}$ ]<sub>i</sub> was measured in a T lymphocyte suspension with a Fura-2 fluorescent probe using a well-established method as described previously<sup>[13]</sup>. Briefly, fresh isolated T lymphocytes ( $1 \times 10^8$  cells/L) were incubated in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing Fura-2/AM (1  $\mu\text{mol}/\text{L}$ ) for 30 min at room temperature. T cells were diluted with RPMI-1640/10% FCS (1:3) and kept at room temperature for 10 min to allow re-equilibration. Cells were washed twice with a  $\text{Ca}^{2+}$ -balanced salt solution (BSS; in mmol/L: NaCl 130, KCl 5,  $\text{MgCl}_2$  1,  $\text{CaCl}_2$  1.5, HEPES 20, glucose 10, pH 7.4), and finally suspended in BSS ( $2 \times 10^6$ – $4 \times 10^6$  cells/mL). [ $\text{Ca}^{2+}$ ]<sub>i</sub> was monitored by using a fluorescence spectrophotometer (RF-5000; Shimadzu, Kyoto, Japan) with dual excitation at 340 nm/380 nm and emission at 500 nm. In order to chelate or remove the residual  $\text{Ca}^{2+}$  in  $\text{Ca}^{2+}$ -free medium, EGTA (50 mmol/L) was added into  $\text{Ca}^{2+}$ -free medium 1 min before determining the release of intracellular  $\text{Ca}^{2+}$ . [ $\text{Ca}^{2+}$ ]<sub>i</sub> was determined from the relation: [ $\text{Ca}^{2+}$ ]<sub>i</sub> =  $K_d \times \text{Sf}380/\text{b}380 (R - R_{\min}) / (R_{\max} - R)$ , where  $K_d$  is 224 nm in the cytoplasmic environment; Sf380/b380 is the ratio of the intensities of the free and bound dye forms at 380 nm;  $R$  is the fluorescence ratio (340 nm/380 nm) of the intracellular Fura-2;  $R_{\max}$  and  $R_{\min}$  are the maximal and minimal fluorescence ratios obtained by addition of Triton X-100 (final con-

centration 0.09%) and EGTA (final concentration 3 mmol/L), respectively<sup>[13]</sup>. Each tested compound was added to the cell suspension when the fluorescence intensity reached the stable state. The measurement of  $[Ca^{2+}]_i$  using Fura-2 is well established in our laboratory<sup>[13,24]</sup>. For every tested compound, we have observed its effect alone on  $[Ca^{2+}]_i$  for up to 15 min and can exclude non-specific effects due to the leakage of Fura-2 dye from cells. All chemicals were purchased from Sigma (USA).

**Probe generation** Human IL-2 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA was ligated into pBluescript II SK (+/-) vectors (Promega, USA), which contain the T3 and T7 RNA promoters. The plasmid DNA was isolated and linearized with *KpnI* or *XbaI*. Then [<sup>32</sup>P-CTP] (Dupont, USA)-labeled hIL-2 and human GAPDH riboprobes were synthesized by using linearized DNA templates, T3 RNA polymerase and the Riboprobe *in vitro* transcription system (Promega, USA). After extraction with phenol/chloroform and chloroform, and precipitation with ethanol, dried probes were dissolved ( $3 \times 10^5$  cpm/mL) in hybridization buffer.

**RPA and RT-PCR analysis** Purified human T cells ( $5 \times 10^6$  cells/mL) were incubated with different blockers for 15 min, at 37 °C, in 5% CO<sub>2</sub>, and then treated with 5 µg/mL ConA for 6 h. Total RNA was extracted using the acid guanidium thiocyanate-phenol-chloroform method<sup>[25]</sup>.

The RPA was performed according to the manual supplied with the RNase protection kit (Ambion, USA). Briefly, a 15 µg total RNA fraction of each sample was hybridized with 1 µL labeled hIL-2 and human GAPDH riboprobes. The hybridized fragments protected from RNase A + T1 digestion were separated by electrophoresis on a standard 8% acrylamide/7M urea sequencing gel. Dried gels were placed on X-ray film for 24 h at -70 °C. The bands on the X-ray film were quantified by densitometry scanning (Koutron IBAS 2.0). The expression level of hIL-2 mRNA was expressed as the ratio of hIL-2/GAPDH.

For RT-PCR, 1 µg of the extracted RNA was used for synthesis of the first-strand cDNA using AMV reverse transcriptase (Promega) according to the manufacturer's instructions. PCR amplification was carried out with 100 ng DNA in a total volume of 50 µL in a Gene Amp 9600 PCR system (Perkin-Elmer, Foster City, CA) for 25 cycles at 94 °C for 2 min, 55 °C (CIC-3) or 57 °C (β-actin) for 1 min, and extension at 72 °C for 1 min. The primers were 5'-ATTAAATGGATATACCCTTTCTTG-3' (sense) and 5'-TTGCAATTGTCAGGTC-TCTTCT-3' (antisense) for CIC-3<sup>[26]</sup>; 5'-GCTACAGCTTCA-CCACCAC-3' (sense) and 5'-TACTCCTGCTTGCTGATCCAC-3' (antisense) for β-actin<sup>[27]</sup>. The amplified products were electrophoresed on 2% agarose gels alongside a

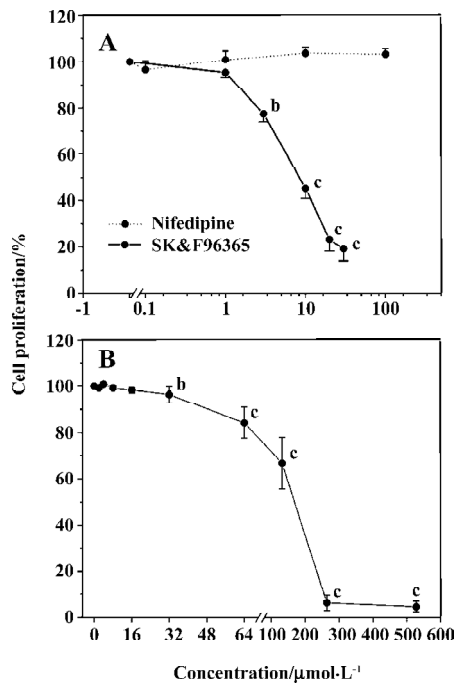
molecular weight marker. The PCR products were 288 and 498 bp in size for CIC3 and β-actin, respectively. Because the primers for β-actin were designed to span two exons and an intron, RT-PCR with β-actin primers controlled for genomic DNA contamination in the source RNA. The linear range of the PCR amplification was determined by carrying out the PCR for varying number of cycles with a fixed quantity of RNA (1 µg), so that the expression levels of CIC-3 could be semi-quantified by densitometry scanning and expressed as the ratio of CIC-3/β-actin.

**Statistical analysis** Results are expressed as mean±SEM. The IC<sub>50</sub> values were estimated in each experiment on the basis of a semi-logarithmic concentration-dependent curve. Data were analyzed with the *t*-test using SPSS11. *P*<0.05 was considered significant.

## Results

**Effects of Ca<sup>2+</sup> and Cl<sup>-</sup> channel blockers on ConA-induced T lymphocyte proliferation** We first investigated whether CRAC and Cl<sup>-</sup> channel blockers affected T cell proliferation using an [<sup>3</sup>H]thymidine incorporation assay. As shown in Figure 1A, SK&F 96365, a common CRAC blocker, significantly inhibited cell proliferation at concentrations of 3 to 30 µmol/L in a concentration-dependent manner, with an IC<sub>50</sub> value of  $9.4 \pm 1.0$  µmol/L, whereas nifedipine (0.1–100 µmol/L), an L-type VDCC inhibitor, did not significantly affect T lymphocyte proliferation (Figure 1A). These data indicate that Ca<sup>2+</sup> entry through CRAC channels, but not VDCC, plays a functional role in T cell proliferation. Next we tested the effects of several Cl<sup>-</sup> channel blockers on human T cell proliferation. As shown in Figure 1B, DIDS significantly inhibited T cell proliferation at concentrations of 32 to 512 µmol/L in a concentration-dependent manner, with an IC<sub>50</sub> value of  $168.0 \pm 3.3$  µmol/L, whereas no inhibitory effects on T cell proliferation were observed in the presence of 100 µmol/L 9-anthracene carboxylic acid (9-AC; *n*=5), 100 µmol/L niflumic acid (NFA; *n*=6) or 400 µmol/L furosemide (*n*=5; *P*>0.05, data not shown). Based on our previous findings that antisense oligonucleotides against CIC-3 decreased [<sup>3</sup>H]thymidine incorporation and prevented T cell proliferation<sup>[15]</sup>, these data suggest that Cl<sup>-</sup> channels, which are sensitive to DIDS, may play a functional role during T lymphocyte proliferation.

Because CRAC-mediated Ca<sup>2+</sup> signaling is the predominant pathway during T cell activation and proliferation<sup>[2]</sup>, the above results prompted us to further study whether Cl<sup>-</sup> channels would affect T cell proliferation through the CRAC-mediated Ca<sup>2+</sup> signaling pathway. Although 2 µmol/L DIDS



**Figure 1.** Effects of  $\text{Ca}^{2+}$  channel blockers and DIDS on ConA (5  $\mu\text{g}/\text{mL}$ )-induced T lymphocyte proliferation. (A) SK&F96365 inhibited ConA (5  $\mu\text{g}/\text{mL}$ )-induced T lymphocyte proliferation in a concentration-dependent manner over the concentration range 1–30  $\mu\text{mol}/\text{L}$  ( $n=6$ ), whereas nifedipine had no significant effect on cell proliferation at concentrations from 0.1 to 100  $\mu\text{mol}/\text{L}$  ( $n=6$ ). The average actual cpm with and without ConA were  $22650 \pm 2173$  cpm and  $2447 \pm 491$  cpm, respectively ( $n=6$ ). (B) Concentration-dependent inhibition of T lymphocyte proliferation by DIDS at concentrations from 2 to 512  $\mu\text{mol}/\text{L}$  ( $n=8$ ). The average actual cpm with and without ConA were  $21476 \pm 2509$  cpm and  $2640 \pm 396$  cpm, respectively ( $n=8$ ). Mean  $\pm$  SEM.  $^{\circ}P < 0.01$  vs no ConA control.

did not significantly affect cell proliferation, the inhibitory effect of SK&F96365 (10  $\mu\text{mol}/\text{L}$ ) on T cell proliferation was enhanced from  $45.4\% \pm 5.3\%$  (without DIDS) to  $67.3\% \pm 4.6\%$  (with 2  $\mu\text{mol}/\text{L}$  DIDS;  $n=6$ ,  $P < 0.01$ ; Table 1). These data suggest that  $\text{Cl}^-$  channels may play a functional role in T cell proliferation through CRAC-mediated  $\text{Ca}^{2+}$  signaling.

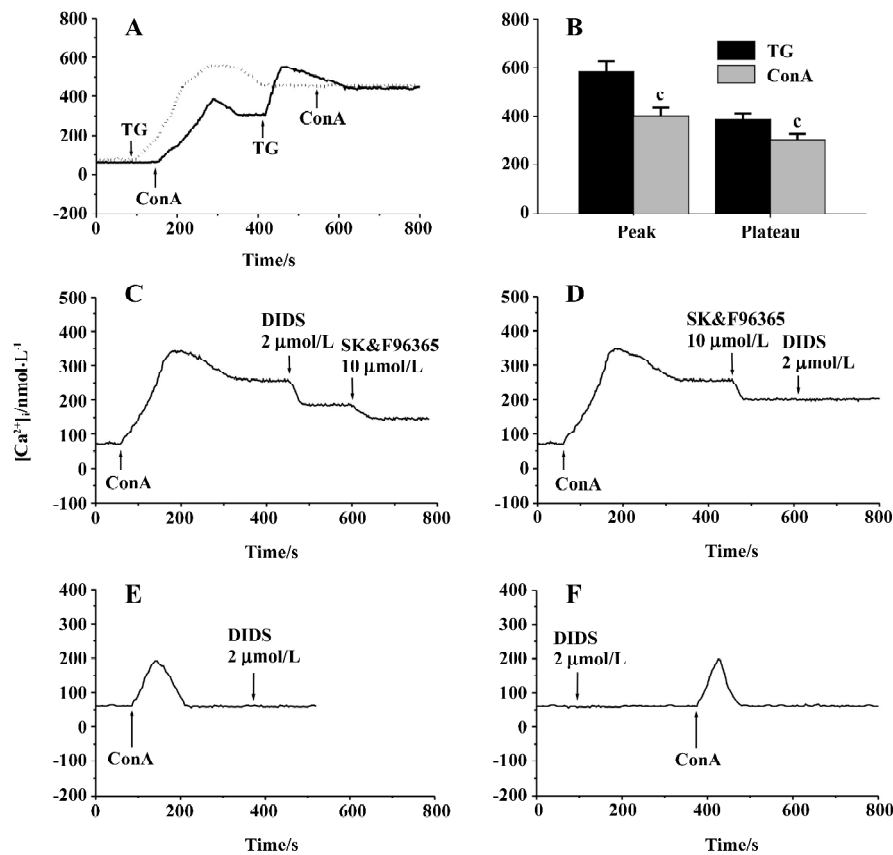
**Effects of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  channel blockers on ConA-induced  $\text{Ca}^{2+}$  entry** Measurement of  $[\text{Ca}^{2+}]_i$  using the Fura-2 probe revealed that the resting  $[\text{Ca}^{2+}]_i$  in human T cells was  $67 \pm 5$  nmol/L ( $n=54$ ), which was not altered by depolarization of T cells with 50 mmol/L KCl ( $66 \pm 10$  nmol/L,  $n=4$ ,  $P > 0.05$ ), indicating a lack of VDCC in the T cell plasma membrane. We then examined the effects of 10  $\mu\text{g}/\text{mL}$  ConA and 2  $\mu\text{mol}/\text{L}$  TG on  $[\text{Ca}^{2+}]_i$  in human T cells, and representative traces are superimposed in Figure 2A. ConA (10  $\mu\text{g}/\text{mL}$ ) caused a transient and then a prolonged increase in  $[\text{Ca}^{2+}]_i$ , which was due to the corresponding release of  $\text{Ca}^{2+}$  from intracellular

**Table 1.** Effects of different treatments on ConA (5  $\mu\text{g}/\text{mL}$ )-induced  $[\text{H}^3]$ thymidine incorporation in human T cells.  $n=6$ . Mean  $\pm$  SEM.  $^{\circ}P < 0.01$  vs ConA-activated control.  $^{\text{f}}P < 0.01$  vs SK&F96365 group.

Groups	$[\text{H}^3]$ thymidine incorporation (cpm)	Inhibitory rate (%)
ConA-activated control	$21577 \pm 2208$	
DIDS (2 $\mu\text{mol}/\text{L}$ )	$20312 \pm 2375$	$0.8 \pm 0.6$
SK&F96365 (10 $\mu\text{mol}/\text{L}$ )	$12007 \pm 1344^{\text{c}}$	$45.4 \pm 5.3^{\text{c}}$
SK&F96365 (10 $\mu\text{mol}/\text{L}$ )+DIDS (2 $\mu\text{mol}/\text{L}$ )	$6413 \pm 1093^{\text{cf}}$	$67.3 \pm 4.6^{\text{cf}}$

stores and the subsequent transmembrane  $\text{Ca}^{2+}$  entry, as shown in previous reports<sup>[3,5,22,28]</sup>. The increase in  $[\text{Ca}^{2+}]_i$  reached steady state within 150 s, and subsequent exposure of the cells to 2  $\mu\text{mol}/\text{L}$  TG caused a further significant increase in  $[\text{Ca}^{2+}]_i$  ( $n=7$ ). However, 10  $\mu\text{g}/\text{mL}$  ConA failed to further increase  $\text{Ca}^{2+}$  influx in T cells whose stores were already depleted by 2  $\mu\text{mol}/\text{L}$  TG ( $n=7$ ). The pattern of the increase in  $[\text{Ca}^{2+}]_i$  stimulated by ConA was similar to that produced by 2  $\mu\text{mol}/\text{L}$  TG except that the peak and the sustained plateau of  $[\text{Ca}^{2+}]_i$  caused by 10  $\mu\text{g}/\text{mL}$  ConA were lower than those caused by 2  $\mu\text{mol}/\text{L}$  TG ( $P < 0.01$ ,  $n=7$ ; Figure 2B). These data indicate that ConA only evokes some  $\text{Ca}^{2+}$  release from TG-sensitive intracellular  $\text{Ca}^{2+}$  stores, which is enough to induce the  $\text{Ca}^{2+}$  influx responsible for T cell activation and proliferation. Our data are consistent with the previous reports showing that lectin induces  $\text{Ca}^{2+}$  influx through the depletion of TG-sensitive intracellular  $\text{Ca}^{2+}$  stores<sup>[2,3,5,29]</sup>. The above proliferation assay demonstrated that 5  $\mu\text{g}/\text{mL}$  ConA produced significant proliferative effects on T cells, but an even higher concentration of ConA (10  $\mu\text{g}/\text{mL}$ ) could not deplete the TG-sensitive intracellular  $\text{Ca}^{2+}$  stores completely; therefore, we used 5  $\mu\text{g}/\text{mL}$  ConA as the stimulant for T cell activation in the subsequent experiments.

We next examined the effects of different  $[\text{Ca}^{2+}]_i$  channel blockers on 5  $\mu\text{g}/\text{mL}$  ConA-induced  $\text{Ca}^{2+}$  entry. When  $\text{Ca}^{2+}$  entry reached a stable state, nifedipine was added to the cell suspensions, which did not affect the ConA-induced  $\text{Ca}^{2+}$  entry for concentrations of 0.1–10  $\mu\text{mol}/\text{L}$  (Table 2), confirming that there are no VDCC in T cells, as described previously<sup>[21]</sup>. As shown in Table 2, SK&F96365 concentration-dependently inhibited ConA (5  $\mu\text{g}/\text{mL}$ )-induced  $\text{Ca}^{2+}$  influx at concentrations of 1–20  $\mu\text{mol}/\text{L}$ . At a concentration of 20  $\mu\text{mol}/\text{L}$ , SK&F96365 inhibited  $\text{Ca}^{2+}$  influx by  $52.9\% \pm 5.4\%$ . SK&F96365 is an inhibitor of  $\text{Ca}^{2+}$  entry through CRAC channels, as well as through VDCC<sup>[23]</sup>. Because T cells do



**Figure 2.** Effects of SK&F96365 and DIDS on ConA-induced increases in  $[Ca^{2+}]_i$  in human T lymphocytes. (A) ConA (10  $\mu\text{g/mL}$ ) and TG (2  $\mu\text{mol/L}$ ) triggered  $[Ca^{2+}]_i$  responses in Fura-2-loaded human T lymphocytes. When changes in  $[Ca^{2+}]_i$  reached a steady state for up to 60 s, ConA or TG was added as indicated by the arrows ( $n=7$  separate experiments). (B) Mean peak and plateau values of  $[Ca^{2+}]_i$  recorded from T cells stimulated by ConA (10  $\mu\text{g/mL}$ ) or TG (2  $\mu\text{mol/L}$ ;  $n=7$ ,  $P<0.01$ ). (C) DIDS 2  $\mu\text{mol/L}$  decreased ConA (5  $\mu\text{g/mL}$ )-induced  $Ca^{2+}$  influx, and the subsequent addition of 10  $\mu\text{mol/L}$  SK&F96365 caused a further decrease in  $[Ca^{2+}]_i$  ( $n=7$  different experiments). (D) ConA (5  $\mu\text{g/mL}$ )-induced  $Ca^{2+}$  influx was inhibited by 10  $\mu\text{mol/L}$  SK&F96365, which was not further altered by the addition of 2  $\mu\text{mol/L}$  DIDS ( $n=7$  different experiments). (E) ConA (5  $\mu\text{g/mL}$ )-induced transient rise in  $[Ca^{2+}]_i$  could not be altered by the subsequent addition of 2  $\mu\text{mol/L}$  DIDS in a  $Ca^{2+}$ -free solution ( $n=6$  different experiments). (F) Pretreatment of cells with 2  $\mu\text{mol/L}$  DIDS failed to induce any change in the subsequent transient rises in  $[Ca^{2+}]_i$  induced by ConA (5  $\mu\text{g/mL}$ ) in  $Ca^{2+}$ -free solution ( $n=5$  different experiments).

not contain VDCC, any effects of SK&F96365 should reflect its effects on CRAC channels. At concentrations of 0.5–2.0  $\mu\text{mol/L}$ , DIDS also decreased  $Ca^{2+}$  influx in a concentration-dependent manner (Table 2). Because DIDS (>3  $\mu\text{mol/L}$ ) began to produce quenching effects on Fura-2 fluorescence, we did not test the effects of higher concentrations of DIDS. The results described so far suggest that DIDS affects  $[Ca^{2+}]_i$ . To investigate whether the inhibition of  $Ca^{2+}$  influx by DIDS is mediated through CRAC channels, the following pharmacological approaches were used. As shown in Figure 2C, 2  $\mu\text{mol/L}$  DIDS caused a 32.3%±5.1% decrease in ConA (5  $\mu\text{g/mL}$ )-induced  $Ca^{2+}$  influx ( $n=7$ ,  $P<0.01$ ), and subsequent exposure of cells to 10  $\mu\text{mol/L}$  SK&F96365 further decreased  $Ca^{2+}$  influx by 19.6%±5.2% ( $n=7$ ,  $P<0.01$ ). However, when 10  $\mu\text{mol/L}$  SK&F96365 caused a decrease in  $Ca^{2+}$  influx by 35.8%±6.2% ( $n=7$ ,  $P<0.01$ ), subsequent addition of 2  $\mu\text{mol/L}$  DIDS brought about no change in  $[Ca^{2+}]_i$  ( $n=7$ ,  $P>0.05$ ; Figure 2D). Within the concentration range of 1–20  $\mu\text{mol/L}$ , SK&F96365 serves as a CRAC channel blocker in T cells<sup>[2,4,23]</sup>, so when the CRAC-mediated  $Ca^{2+}$  influx was prevented by 10  $\mu\text{mol/L}$  SK&F96365, 2  $\mu\text{mol/L}$  DIDS did not induce any

change in  $[Ca^{2+}]_i$ , suggesting that DIDS inhibits the ConA-induced  $Ca^{2+}$  influx through interfering with the CRAC-mediated pathway. We also tested whether DIDS directly affects ConA (5  $\mu\text{g/mL}$ )-induced  $Ca^{2+}$  release. Figure 2E shows that in  $Ca^{2+}$ -free solution (following the removal of  $CaCl_2$  and after addition of 200  $\mu\text{mol/L}$  EGTA), ConA (5  $\mu\text{g/mL}$ ) evoked a transient increase in  $[Ca^{2+}]_i$ , which was due to  $Ca^{2+}$  release from intracellular  $Ca^{2+}$  stores, but the subsequent application of 2  $\mu\text{mol/L}$  DIDS failed to induce any change in  $[Ca^{2+}]_i$  ( $n=6$ ,  $P>0.05$ ; Figure 2E). In addition, pretreatment of cells with 2  $\mu\text{mol/L}$  DIDS did not induce any change in  $[Ca^{2+}]_i$  in  $Ca^{2+}$ -free solution and further failed to alter subsequent ConA-induced  $Ca^{2+}$  release ( $n=5$ ,  $P>0.05$ , Figure 2F). These data indicate that 2  $\mu\text{mol/L}$  DIDS had no direct effect on ConA-induced  $Ca^{2+}$  release or resting  $[Ca^{2+}]_i$ .

**Effects of  $Ca^{2+}$  and Cl<sup>-</sup> channel blockers on ConA-induced IL-2 mRNA expression** In T cells, CRAC-mediated sustained increase in  $[Ca^{2+}]_i$  activates several transcription factors, including NF- $\kappa$ B, Oct/OAP, and NF-AT, then initiates the transcription of IL-2 mRNA, which results in IL-2 production and finally proliferation of T cells<sup>[2]</sup>. RPA analy-

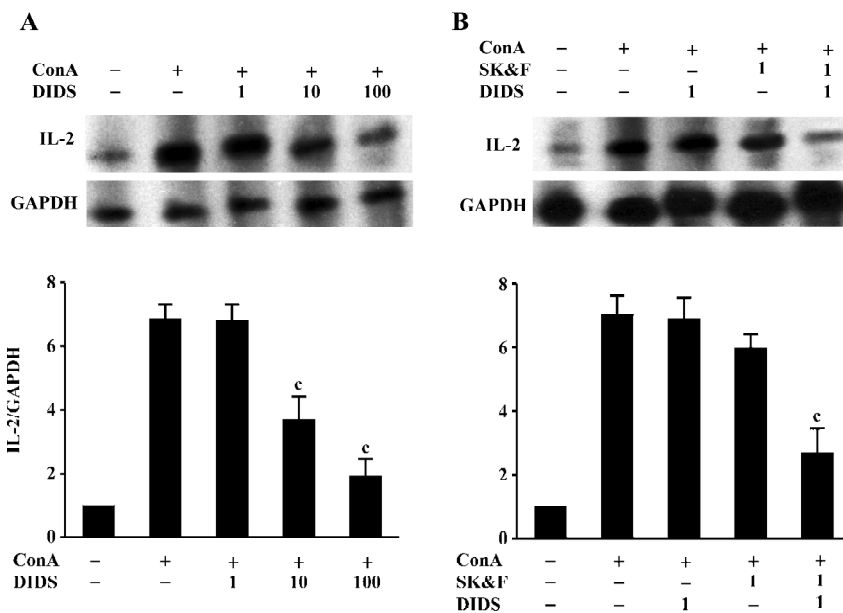
**Table 2.** Effects of nifedipine, SK&F96365 and DIDS on ConA (5  $\mu\text{g}/\text{mL}$ )-induced  $\text{Ca}^{2+}$  influx in human T lymphocytes. Mean $\pm$ SEM. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs the corresponding pretreatment groups.

	<i>n</i>	$[\text{Ca}^{2+}]_i$ (nmol/L)	Inhibition of $\text{Ca}^{2+}$ influx (%)
Pretreatment	5	256.6 $\pm$ 28.8	
Nifedipine ( $\mu\text{mol}/\text{L}$ )			
0.1	5	249.1 $\pm$ 27.5	
1	5	252.3 $\pm$ 26.4	
10	5	254.7 $\pm$ 24.1	
Pretreatment	6	250.2 $\pm$ 24.9	
SK&F96365 ( $\mu\text{mol}/\text{L}$ )			
1	6	253.1 $\pm$ 25.4	1.6 $\pm$ 2.7
3	6	239.4 $\pm$ 20.9 <sup>b</sup>	8.9 $\pm$ 3.5 <sup>c</sup>
10	6	193.3 $\pm$ 12.7 <sup>c</sup>	33.6 $\pm$ 6.1 <sup>c</sup>
20	6	163.8 $\pm$ 17.5 <sup>c</sup>	52.9 $\pm$ 5.4 <sup>c</sup>
Pretreatment	8	242.3 $\pm$ 26.7	
DIDS ( $\mu\text{mol}/\text{L}$ )			
0.25	8	235.7 $\pm$ 28.8	3.8 $\pm$ 5.1
0.5	8	209.9 $\pm$ 16.3 <sup>b</sup>	18.1 $\pm$ 4.9 <sup>c</sup>
1	8	197.3 $\pm$ 17.0 <sup>c</sup>	28.3 $\pm$ 6.7 <sup>c</sup>
2	8	180.0 $\pm$ 13.0 <sup>c</sup>	30.5 $\pm$ 5.4 <sup>c</sup>

The resting  $[\text{Ca}^{2+}]_i$  of human T lymphocytes was 72 $\pm$ 7 nmol/L ( $n=14$ ).

sis demonstrated that ConA (5  $\mu\text{g}/\text{mL}$ ) increased IL-2 mRNA expression in human T cells after 6 h of incubation (662% $\pm$ 45%

increase;  $n=7$ ,  $P<0.01$ ). To examine the effects of ion channel blockers on ConA-stimulated IL-2 mRNA expression, cells were incubated with the blockers for 30 min prior to exposure to ConA. As shown in Figure 3A, 10 and 100  $\mu\text{mol}/\text{L}$  DIDS caused a decrease in ConA-stimulated hIL-2 mRNA expression, with inhibitory rates of 37% $\pm$ 8% and 69% $\pm$ 7%, respectively ( $n=4$ ,  $P<0.01$  vs ConA-stimulation control), whereas 1  $\mu\text{mol}/\text{L}$  DIDS had no effect on IL-2 mRNA expression in stimulated T cells ( $n=4$ ,  $P>0.05$  vs ConA-stimulation control). Consistent with previous reports<sup>[4]</sup>, SK&F96365 also decreased IL-2 mRNA expression. We observed that 1  $\mu\text{mol}/\text{L}$  SK&F96365 decreased IL-2 mRNA expression by 12% $\pm$ 5% ( $n=5$ ,  $P>0.05$  vs ConA-treatment group; Figure 3B). The inhibition of ConA-stimulated hIL-2 mRNA expression was significantly enhanced by the combination of 1  $\mu\text{mol}/\text{L}$  SK&F96365 and 1  $\mu\text{mol}/\text{L}$  DIDS ( $n=5$ ,  $P<0.01$  vs the corresponding ConA plus blocker group). The inhibitory rates for DIDS, SK&F96365, and SK&F96365 plus DIDS were 2% $\pm$ 1%, 12% $\pm$ 5%, and 66% $\pm$ 9%, respectively. It has been demonstrated that naive T cell stimulation induces IL-2 mRNA, which is present for at least 5 h. This shortburst of IL-2 mRNA allows for the secretion of functional levels of IL-2 protein that are sufficient to induce naive T cells to enter the cell cycle<sup>[30]</sup>. In the present study, we harvested total mRNA after the co-incubation of cells with the tested compounds for 6 h. Thus, comparing the effects of both channel blockers on IL-2 mRNA expression could reflect the functional role of the  $\text{Cl}^-$  channels during  $\text{Ca}^{2+}$ -dependent T cell proliferation signaling cascades.



**Figure 3.** Effects of SK&F96365 and DIDS on ConA-stimulated IL-2 mRNA expression in human T lymphocytes as determined by the RPA. (A) The upper bands show that DIDS significantly inhibited IL-2 mRNA expression at concentrations of 10 and 100  $\mu\text{mol}/\text{L}$ . No significant changes were observed at a concentration of 1  $\mu\text{mol}/\text{L}$ . Similar results were observed in 5 experiments. The corresponding IL-2/GAPDH mRNA ratios obtained from densitometric analysis are shown in the lower bar graph. (B) The upper bands show that the combined application of DIDS (1  $\mu\text{mol}/\text{L}$ ) and SK&F96365 (1  $\mu\text{mol}/\text{L}$ ) produced a much stronger inhibition of ConA-stimulated IL-2 mRNA expression than that of each alone. Similar results were obtained in 5 experiments. The lower bar graph shows the corresponding IL-2/GAPDH mRNA ratios obtained from densitometric analysis of the upper bands. Mean $\pm$ SEM. <sup>c</sup> $P<0.01$  vs ConA treatment group.

**Effects of Ca<sup>2+</sup> and Cl<sup>-</sup> channel blockers on ConA-induced CIC-3 expression** Our recent studies support the notion that CIC-3 may be the potential functional player during T cell proliferation<sup>[15,16]</sup>. Therefore, we examined the effects of DIDS and its synergistic effect together with SK&F96365 on CIC-3 mRNA expression. As shown in Figure 4A, using primers specific to the human cDNA sequence of CIC-3, bands corresponding to the expected fragment size (248 bp) were obtained from the total RNA prepared from human T lymphocytes exposed to different treatments. CIC-3 transcripts were found in all samples, and were increased by ConA-treatment, which was consistent with the CIC-3 protein expression described in human T lymphocytes using Western blot analysis<sup>[16]</sup>. As summarized in Figure 4B using CIC-3/ $\beta$ -actin ratios, the exposure of T cells to 1  $\mu$ mol/L DIDS and 1  $\mu$ mol/L SK&F96365 decreased ConA-induced CIC-3

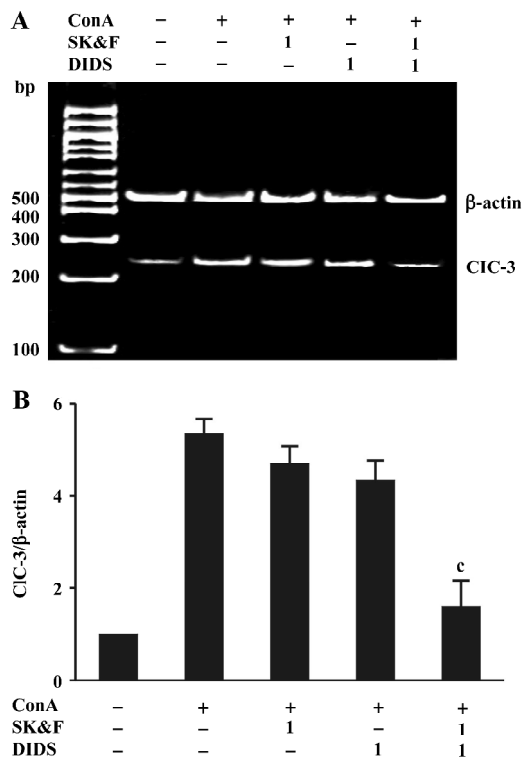
mRNA expression by 16% $\pm$ 6% and 9% $\pm$ 4%, respectively ( $n=6$ ,  $P>0.05$  vs ConA-treatment group). Moreover, the combination of 1  $\mu$ mol/L DIDS and 1  $\mu$ mol/L SK&F96365 acted synergistically to diminish CIC-3 mRNA expression by 64% $\pm$ 10% ( $n=6$ ,  $P<0.05$  vs the corresponding ConA plus blocker group). The  $\beta$ -actin bands corresponding to 500 bp are the same in all lanes. These data suggest that the effect of DIDS on the ConA-induced CIC3 message may be linked to CRAC-mediated signaling during T cell activation.

**Discussion**

The present study demonstrates the following events: (1) DIDS, a chloride channel blocker, inhibits ConA-induced human T lymphocyte proliferation in a concentration-dependent manner, but 9-AC, NFA, and furosemide have no significant effect on T cell proliferation induced by ConA; (2) when combined with SK&F96365, DIDS enhances the inhibitory effect of SK&F96365 on ConA-induced IL-2 mRNA expression and T cell proliferation; (3) both DIDS and SK&F96365 decreased ConA-induced Ca<sup>2+</sup> influx, but the mechanisms used by the two inhibitors may be different; (4) a combination of DIDS and SK&F96365 enhanced the inhibition of ConA-induced CIC-3 mRNA expression relative to the effect of each alone.

Fura-2 fluorescence analysis showed that ConA-treatment did not further increase Ca<sup>2+</sup>-influx in T cells whose stores had been already depleted by TG. This observation agrees with the recognized idea that CRAC channel is solely responsible for Ca<sup>2+</sup>-influx during lectin-induced T cell activation<sup>[2]</sup>. Thus, the effects of SK&F96365 on ConA-induced T cell activation and proliferation can be attributed to the inhibition of Ca<sup>2+</sup>-influx through CRAC channel.

A growing number of reports suggest that Cl<sup>-</sup> channels play a functional role in the cellular proliferation process in T cells and other cell types<sup>[12,13,17,19]</sup>, which was further supported by our present work examining the effects of DIDS on several pivotal steps during T cell proliferation, including early Ca<sup>2+</sup> signaling and IL-2 mRNA expression as well as later cell proliferation. In our present and previous studies, DIDS has been shown to prevent cell proliferation in T lymphocytes stimulated by lectins (ConA) and in rat aortic smooth muscle cells stimulated by ET-1<sup>[13,18]</sup>. However, it should be noted that DIDS is also an effective blocker of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers, ATP-sensitive K<sup>+</sup> channels, and can bind to other proteins<sup>[31,32]</sup>. Due to the lack of specific chloride channel blockers, it is difficult to confirm whether Cl<sup>-</sup> channels play a functional role in cell proliferation just using Cl<sup>-</sup> channel blockers. To exclude several ion transports from the potential candidate list for cell proliferation, we first com-



**Figure 4.** Effects of SK&F96365 and DIDS on CIC-3 mRNA expression. The amplified PCR products corresponding to the predicted size of the cDNA segment (248 bp for CIC-3 and 498 bp for  $\beta$ -actin) were detected in all samples (A). ConA (5  $\mu$ g/mL) significantly stimulated CIC-3 mRNA expression; neither DIDS (1  $\mu$ mol/L) nor SK&F96365 (1  $\mu$ mol/L) significantly altered CIC-3 mRNA expression; DIDS (1  $\mu$ mol/L) plus SK&F96365 (1  $\mu$ mol/L) significantly evoked a decrease in CIC-3 mRNA expression. The corresponding CIC-3/ $\beta$ -actin mRNA ratios were obtained from densitometric analysis of the upper bands (B). Similar results were observed in 6 different experiments. Mean $\pm$ SEM. <sup>c</sup> $P<0.01$  vs ConA-treatment group.

pared the effects of chemically different Cl<sup>-</sup> channel blockers on ConA-induced T lymphocyte proliferation. As shown in our previous studies<sup>[13,18]</sup>, it appears that the effect of DIDS and NPPB on proliferation is not related to Ca<sup>2+</sup>-activated or maxi Ca<sup>2+</sup> channels, because the Cl<sup>-</sup> channel inhibitors NFA (Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel blocker), SITS (Ca<sup>2+</sup>-activated and maxi Cl<sup>-</sup> channel blocker), and IAA-94 (Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel blocker) did not change the effect of ConA on T cell proliferation. Among the CIC family members identified in immune cells (CIC-2, 3, 4, 5), CIC-2 has been excluded from the candidate lists for cellular proliferation<sup>[17]</sup>. Another potential candidate may be the CIC-3 Cl<sup>-</sup> channel. Abundant expression of CIC-3 mRNA and protein has been detected in T lymphocytes<sup>[16,26]</sup>. Our previous work provided compelling evidence of the functional role of CIC-3 protein in ConA-induced T cell proliferation using antisense oligonucleotides against CIC-3. We found that the downregulation of CIC-3 by antisense, but neither sense or missense, oligonucleotides against CIC-3 not only inhibited ConA-induced CIC-3 expression but also decreased [<sup>3</sup>H]thymidine incorporation and prevented T cell proliferation<sup>[15,16]</sup>. The Cl<sup>-</sup> current recorded in T cells shares similar pharmacological properties with the expressed CIC-3 currents in mammalian cell lines, such as sensitivity to Cl<sup>-</sup> channel blockers including DIDS, NPPB and tamoxifen<sup>[33,34]</sup>. These studies, along with the present work examining the effects of Cl<sup>-</sup> channel blockers on several key signaling steps during T cell proliferation, strongly suggest that CIC-3 channels may play functional roles during T cell proliferation signaling cascades. Although it is still unclear whether the CIC-3 protein is a volume-regulated channel or a component thereof in some cell types<sup>[35,36]</sup>, the involvement of CIC-3 protein in cell proliferation has received strong support from studies carried out in our laboratory and other independent studies<sup>[12]</sup>. On the other hand, the present study could not indicate whether CIC-3 is solely responsible for Cl<sup>-</sup> channel activation by ConA in T cells. We have not accepted Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels ( $I_{Cl(Ca)}$ ) as potential candidates, because NFA, a very common blocker of  $I_{Cl(Ca)}$  did not alter T cell activation. Elucidating the exact nature of Cl<sup>-</sup> channel activation in T cell proliferation will require further study.

In addition to their direct effect on T cell activation, Cl<sup>-</sup> channels may participate in T cell activation through the regulation of Ca<sup>2+</sup> signaling. Although CRAC channels are not opened directly by depolarization, Ca<sup>2+</sup> ions entering the cell through CRAC channels feed back to inhibit the channel's activity by reducing the membrane potential. K<sup>+</sup> and Cl<sup>-</sup> channels have been found to assist with Ca<sup>2+</sup> entry by maintaining a sufficiently negative membrane potential<sup>[37]</sup>.

Therefore, it is reasonable to suggest that Cl<sup>-</sup> channels may be involved in T cell activation by regulation of the CRAC channels. Our data also indicated that ConA-induced Ca<sup>2+</sup> release was not significantly changed by DIDS, which further supports the finding that DIDS at low concentrations has no effect on Ca<sup>2+</sup> release in some cell types<sup>[38,39]</sup>. Thus, it seems that the effect of DIDS on T cell activation is due to the inhibition of CRAC-induced Ca<sup>2+</sup> entry. This appears to be the first description at different molecular levels of the interaction between Cl<sup>-</sup> channels and CRAC-mediated Ca<sup>2+</sup> signaling during T cell activation. Although the link between Cl<sup>-</sup> channels and CRAC-mediated Ca<sup>2+</sup> influx cannot be fully explained by the quenching effects of DIDS (>3 μmol/L) on Fura-2 fluorescence, the concentration range of DIDS that produced effects on CIC-3, IL-2 mRNA expression and cell proliferation agree with the characterization of Cl<sup>-</sup> channels determined in other studies<sup>[19,28,34,40]</sup>.

Although a sustained Ca<sup>2+</sup> plateau is the critical trigger inducing downstream IL-2 gene expression and cell proliferation in ConA-activated T cells, how the specific information embedded in the amplitude, duration and kinetics of Ca<sup>2+</sup> signals is transmitted to downstream selective gene expression in T cells remains an unanswered key question in cell signaling<sup>[8]</sup>. In addition, the various sizes of commonly distributing Ca<sup>2+</sup> oscillations also adds more complexity to Ca<sup>2+</sup> signaling in T cells<sup>[8]</sup>. Therefore, T lymphocyte Ca<sup>2+</sup> signals are more than a binary switch in T cell activation, and different patterns of Ca<sup>2+</sup> signals may transmit according to the specific patterns of gene expression, which will elicit final selective responses in activated T cells. The emerging complex Ca<sup>2+</sup> signaling in T cell activation may also help to explain the inconsistencies in effective DIDS concentrations in the present study. A concentration of 2 μmol/L DIDS significantly inhibited CRAC-induced Ca<sup>2+</sup> entry, but had no effect on IL-2 mRNA expression and final T cell proliferation. It is reasonable to assume that DIDS-sensitive Cl<sup>-</sup> channels play a time-dependent functional role during the whole process of cellular proliferation. Acute exposure to DIDS (less than a few seconds) at a low concentration may only transiently reduce Ca<sup>2+</sup> signaling and fail to affect later gene expression and final cell proliferation; whereas long-term exposure (up to a number of hours) to higher concentrations of DIDS may cause complete inhibition of specific Ca<sup>2+</sup> signaling, leading to the inhibition of specific gene expression and final T cell proliferation.

In summary, the results of our present study suggest that DIDS-sensitive Cl<sup>-</sup> channels may play a functional role in ConA-stimulated, Ca<sup>2+</sup>-dependent IL-2 mRNA expression and cell proliferation in T cells through CRAC-mediated Ca<sup>2+</sup>



entry. Definitive molecular determination of the DIDS-sensitive Cl<sup>-</sup> channels will require gene targeting experiments combined with more specific pharmacological tools. CIC-3 may be responsible for the DIDS-sensitive Cl<sup>-</sup> channel, which may serve as a novel interfering target during the signaling transduction of T cell activation and proliferation.

### Footnotes

Abbreviations used in this paper: ConA, concanavalin A; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular free calcium concentration; CRAC, Ca<sup>2+</sup>-release-activated Ca<sup>2+</sup>; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid; SK&F96365, 1- $\beta$ -[3-(4-methoxyphenyl)propoxyl]-4-methoxyphenethyl]-1*H*-imidazole; TG, thapsigargin; NPPB, 5-nitro-2-(5-phenylpropylamino)-benzoate; 9-AC, 9-anthracene carboxylic acid; NFA, niflumic acid; STIS, 4-acetamido-4-isocyanostilbene-2,2-disulphonic acid; IAA-94, Indanyloxyacetic acid; RPA, RNase protection assay; VDCC, voltage-dependent Ca<sup>2+</sup> channel.

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