

# Inhibition by SK&F 96365 of Ca<sup>2+</sup> current, IL-2 production and activation in T lymphocytes

S. Clare Chung, Thomas V. McDonald & <sup>1</sup>Phyllis Gardner

Department of Molecular Pharmacology and Medicine, Stanford University School of Medicine, Stanford, California 94305-5332, U.S.A.

1 By use of whole cell patch-clamp and Indo-1 fluorescence studies of the Jurkat T leukaemic cell line, we show that the new organic antagonist of receptor-mediated Ca<sup>2+</sup> entry, SK&F 96365, inhibits the T cell Ca<sup>2+</sup> current in a dose-dependent fashion, with an IC<sub>50</sub> of 12 μM.

2 SK&F 96365 also inhibits [<sup>3</sup>H]-thymidine incorporation and interleukin-2 (IL-2) synthesis in peripheral blood lymphocytes.

3 SK&F 96365 has no effect on Ca<sup>2+</sup> stores release or K<sup>+</sup> channels.

4 This is the first account of an organic inhibitor of the T cell Ca<sup>2+</sup> current. The ability of SK&F 96365 to inhibit IL-2 synthesis and cell proliferation suggests that a new class of related Ca<sup>2+</sup> channel blockers can be developed as immunosuppressive agents.

**Keywords:** T lymphocytes; Ca<sup>2+</sup> channels; SK&F 96365

## Introduction

The early events of T cell activation include an elevation in intracellular Ca<sup>2+</sup>, which consists of two phases, a transient release from intracellular stores and a sustained influx across the plasma membrane (Gardner, 1989). The latter phase of Ca<sup>2+</sup> influx is critical for full T cell activation, since either treatments that reduce Ca<sup>2+</sup> influx (Jy *et al.*, 1989; Mignery *et al.*, 1992) or T cell receptor (TCR) mutants with submaximal and transient changes in [Ca<sup>2+</sup>]<sub>i</sub> in response to stimulation (Goldsmith *et al.*, 1989) do not produce the autocrine growth factor interleukin-2 (IL-2), as required for progression through the cell cycle (Crabtree, 1989).

The mechanism by which Ca<sup>2+</sup> traverses the plasma membrane has been the subject of many studies. Plasmalemmal Ca<sup>2+</sup> channels can be divided into two broad categories; voltage-gated and non-voltage gated. Voltage-gated channels are found in nervous, endocrine, cardiac and skeletal tissue (Nowycky *et al.*, 1985; Tsien *et al.*, 1991). The L-type, or dihydropyridine-sensitive, voltage-gated channels have been well characterized and are the target of the clinically used Ca<sup>2+</sup> channel blockers such as verapamil, diltiazem, nifedipine and nifedipine (Lee & Tsien, 1983; Brown *et al.*, 1984; Hess & Tsien, 1984) but early patch clamp studies ruled out the presence of classical voltage-gated Ca<sup>2+</sup> channels in the T cell membrane (DeCoursey *et al.*, 1984; Matteson & Deutsch, 1984). Instead, one or more non-voltage gated Ca<sup>2+</sup> channels have been found in lymphocytes by means of patch clamp recording (Kuno *et al.*, 1986; Kuno & Gardner, 1987; Lewis & Cahalan, 1989; McDonald *et al.*, 1993). Whole-cell studies suggest that the major source of Ca<sup>2+</sup> influx flows through a highly Ca<sup>2+</sup>-selective, inwardly rectifying, Cd<sup>2+</sup> and Ni<sup>2+</sup> sensitive channel (Lewis & Cahalan, 1989; McDonald *et al.*, 1993; Zweifach & Lewis, 1993) that is activated either after intracellular generation of InsP<sub>3</sub> (Lewis & Cahalan, 1989; McDonald *et al.*, 1993) or after the application of endoplasmic reticulum Ca<sup>2+</sup> ATPase inhibitors such as thapsigargin (TG) (Zweigach & Lewis, 1993; Premack *et al.*, 1994). The characteristics of the current, as well as its activation by various modalities that deplete intracellular Ca<sup>2+</sup> stores, suggest that it is in the same category as *I*<sub>crac</sub> (for Ca<sup>2+</sup> release associated current) first described in mast cells (Hoth & Penner, 1992). This current is activated by a 'capacitative' mechanism (Putney, 1990), in which depletion of the endo-

plasmic reticulum Ca<sup>2+</sup> stores serves as a trigger for Ca<sup>2+</sup> influx by an as yet unidentified mechanism. Though current which flows in response to depletion of stores is blocked by inorganic ions including Cd<sup>2+</sup>, Ni<sup>2+</sup>, and La<sup>3+</sup>, there are as yet no reported organic inhibitors of *I*<sub>crac</sub>.

We undertook the present study to determine if SK&F 96365 (1-[*b*-[3-(*p*-methoxyphenyl)-propyloxy-*p*-methoxyphenyl]-1H-imidazole hydrochloride), a receptor-mediated Ca<sup>2+</sup> entry inhibitor in platelets and endothelial cells (Merritt *et al.*, 1990; Mason *et al.*, 1991; Graier *et al.*, 1992) inhibits the *I*<sub>crac</sub> type Ca<sup>2+</sup> current in Jurkat T lymphoblasts. To activate the Ca<sup>2+</sup> current in Jurkat cells, we used either the mitogen phytohaemagglutinin (PHA), monoclonal antibody to the T cell receptor (mAb C305), carbachol (CCh) stimulation of Jurkat cells transfected with human muscarinic receptor 1 (JHM1 cells; 12), or the microsomal Ca<sup>2+</sup> ATPase inhibitor thapsigargin (TG). Each of these methods of receptor stimulation have been shown to activate the characteristic, inwardly rectifying, Ca<sup>2+</sup>-selective whole-cell current (Lewis & Cahalan, 1989; McDonald *et al.*, 1993; Zweifach & Lewis, 1993; Premack *et al.*, 1994). We also assessed effects of SK&F 96365 on T cell function by monitoring cellular proliferation and IL-2 synthesis. We show here that SK&F 96365 inhibits T cell Ca<sup>2+</sup> current, mitogen or thapsigargin-induced cellular proliferation, and IL-2 synthesis with a similar concentration-dependence. This suggests that a new class of related Ca<sup>2+</sup> channel blockers may be developed to inhibit the *I*<sub>crac</sub> class of Ca<sup>2+</sup> channels and that this class of drugs may have therapeutic potential as immunosuppressants.

## Methods

### Cell lines

Jurkat E6-1 cells of malignant T-lymphocyte cell line were grown at 37°C (5% CO<sub>2</sub>) in media RPMI 1640 (Irvine Scientific) supplemented with 10% foetal bovine serum (Hyclone) and L-glutamine. A stable cell line transfected with the human muscarinic receptor type 1 (De Coursey *et al.*, 1984) was kindly provided by Dr A. Weiss (University of California San Francisco Medical Center). Transfected cells were grown in the above media with the addition of G418 (2 mg ml<sup>-1</sup>) to maintain selection of HM1 + populations.

<sup>1</sup> Author for correspondence.

HT-2 lymphocytes, which are dependent on human IL-2 for growth and which were used in the IL-2 assay, were a gift from Dr C. Clayberger (Stanford University).

### Peripheral blood lymphocytes

Peripheral blood lymphocytes were obtained by standard procedures from a healthy volunteer on the day of the experiment. Briefly, blood samples were prepared as follows. First the sample was layered onto Ficoll-Hypaque separation media. This eluent was centrifuged at 2000 r.p.m. for 15 min and peripheral blood lymphocyte fraction was collected. Excess Ficoll was washed from cell fraction and centrifuged at 1200 r.p.m. for 10 min. The pellet was resuspended in PBS buffer and centrifuged at 800 r.p.m. to separate the lymphocytes from the platelets. Cells were washed several times with PBS buffer. The isolated lymphocytes were placed in a media RPMI 1640 with 10% foetal bovine serum.

### Materials

SK&F 96365 was a gift from Dr Janet E. Merritt from Smith & Kline and Beecham Pharmaceuticals. MAb C305 was a gift from Dr A. Weiss (University of California at San Francisco). Indo-1/AM and Indo-1/K salt were obtained from Molecular Probes.

### Indo-1/AM loading and population $[Ca^{2+}]_i$ measurements

Cells were loaded with 2.5  $\mu$ M Indo-1/AM in complete growth medium at 30°C for 45 min followed by successive washes to remove unincorporated dye. The cells were then resuspended ( $5 \times 10^5$  cells per 2 ml) in solution of the following composition (mM): NaCl 137, KCl 5.8, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 2.5, glucose 5 and HEPES 10, pH = 7.4. Cell suspensions were studied in acrylic cuvettes with constant stirring at 30°C. Fluorescence measurements for the determination of  $[Ca^{2+}]_i$  were made in a spectrofluorimeter (SLM 8000/Aminco) with excitation wavelength of 350 nm and dual simultaneous emission fluorescence detection at 405 nm and 464 nm. Agonists were introduced by pipette addition to the cuvettes. Final  $[Ca^{2+}]_i$  values were calculated from the emission ratio with the standard equation (Grynkewicz *et al.*, 1985). The  $R_{max}$  value was obtained following treatment of cell suspensions with 10  $\mu$ M ionomycin. The  $R_{min}$  value was obtained with a calibration solution (mM): KCl 140, MgCl<sub>2</sub> 2, EGTA 10, HEPES 20 calibration solution which contained Indo-1 K salt 1  $\mu$ M.

### Electrophysiology

Cells were placed in a acrylic/polystyrene perfusion chamber mounted on the stage of an inverted microscope (Nikon Diaphot). Initial extracellular solution was mM: Tris-Cl 170, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 2.5, glucose 5 and HEPES 10, pH = 7.4, at room temperature (20°C). Intracellular pipette solution contained mM: Cs-glutamate 80 (or Cs-aspartate 80), CsCl 8, MgCl<sub>2</sub> 2, EGTA 20 and HEPES 20, pH = 7.3. The estimated  $E_{rev Cl^-} = -71$  mV with these solutions. Carbachol (100–200  $\mu$ M), thapsigargin (100 nM), and monoclonal antibody to TCR (1/10<sup>4</sup> dilution) addition and changes in extracellular ion concentrations were made by use of a perfusion system which exchanged the chamber bath solution within 30–35 s. Solutions for ion substitution experiments were adjusted to avoid changes in osmolality.

For studies testing the effect of SK&F 96365 on K<sup>+</sup> currents, the extracellular solution was (mM): NaCl 130, CaCl<sub>2</sub> 10, MgCl<sub>2</sub> 2, KCl 5 and HEPES 10, pH = 7.3. The intracellular pipette solution (mM) was: KCl 140, MgCl<sub>2</sub> 2, EGTA 10 and HEPES 10, pH 7.3.

The whole-cell configuration of the patch clamp technique was used to record plasma membrane currents (Hamil *et al.*,

1981). Glass pipettes with a tip resistance of 1–3 MegOhms was used. An Axopatch-1C patch-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.) and a 80386-based PC computer with a 12-bit AD/DA converter (Scientific solutions) were used for membrane voltage clamp protocols and whole-cell current recording. Data was stored in digital form on floppy diskettes for later analysis with pClamp software (Axon Instruments). Holding potential was maintained at –70 mV and voltage clamp protocols included steps (from –125 mV to +50 mV in 25 mV increments) to monitor steady-state currents and repetitive voltage ramps at 0.75 mV ms<sup>-1</sup> (from –125 mV to +50 mV) to generate current-voltage (*I*–*V*) curves. For studies of K<sup>+</sup> current, holding potential was maintained at –70 mV and voltage clamp protocols included steps from –125 mV to +50 mV in 25 mV increments.

### Cell proliferation assay

Cells ( $3 \times 10^5$  cells/well) were placed in a 96-well plate and incubated with PHA (20  $\mu$ g ml<sup>-1</sup>) or thapsigargin (100 nM) for 24 h: 1  $\mu$ Ci of [<sup>3</sup>H]-thymidine was then added to each well. Cells were incubated for 24 h and harvested onto a filter paper. The filter papers were placed in a counting vial and counted in a beta radiation counter (1500-TRI-CARB liquid scintillation analyzer by Packard).

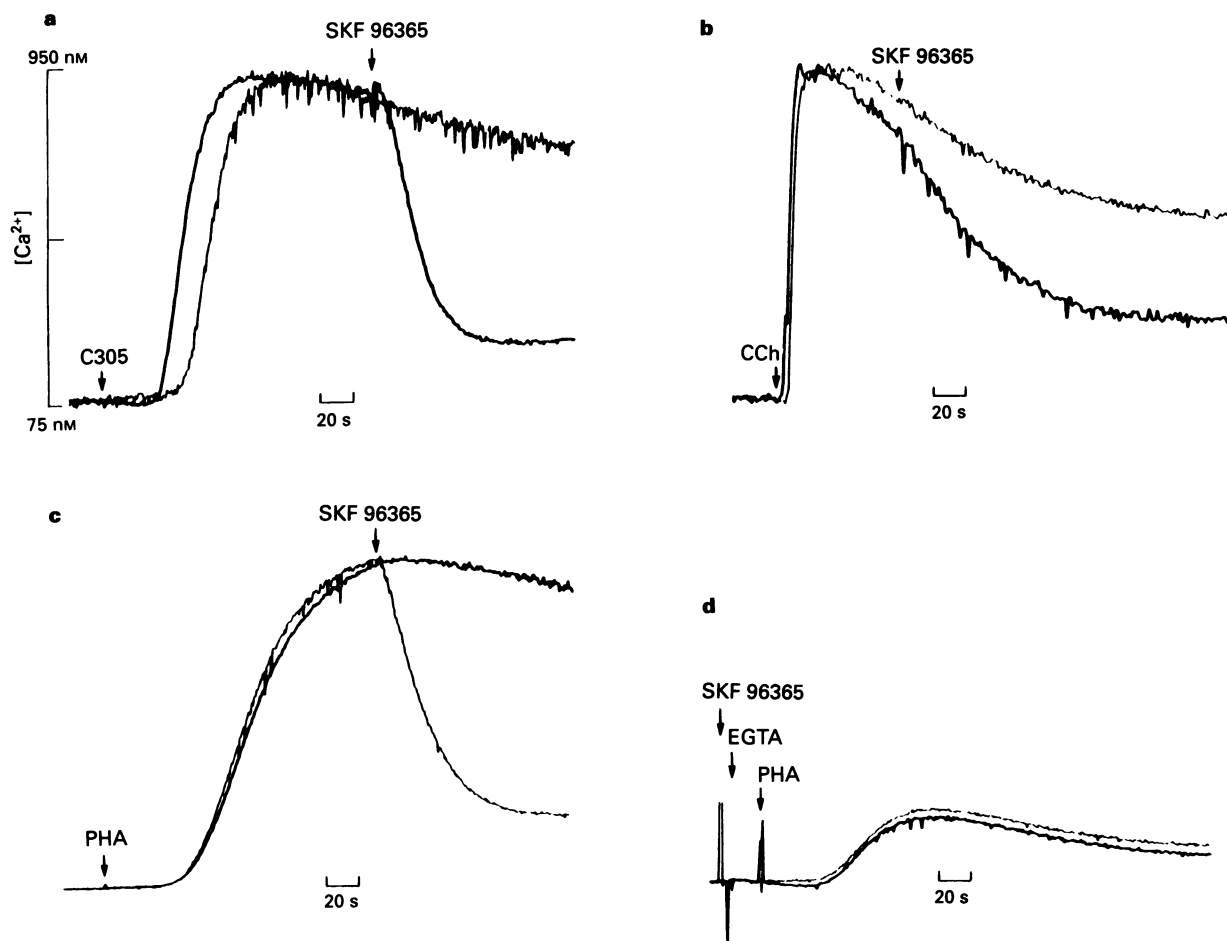
### IL-2 assay

Peripheral blood lymphocytes ( $5 \times 10^6$  cells ml<sup>-1</sup>) were incubated with PHA (20  $\mu$ g ml<sup>-1</sup>) for 18 h and the supernatant was collected from these cells. The supernatant was then added to  $5 \times 10^3$  HT-2 cells per well and cells were cultured for 24 h at 37°C. [<sup>3</sup>H]-thymidine (1  $\mu$ Ci) was then added to each well. Cells were incubated for 4 h and then harvested onto a filter paper. The papers were counted in a beta radiation counter (1500-TRI-CARB liquid scintillation analyzer by Packard). Different concentrations of human interleukin-2 (IL-2; Sigma) were added to HT-2 cells to create a standard curve. Control experiments in which different concentrations of SK&F 96365 were added with IL-2 to HT-2 cells, confirmed that SK&F 96365 had no effect on the bioassay.

## Results

### Effects of SK&F 96365 on agonist-stimulated $[Ca^{2+}]_i$ responses in Indo-1/AM-loaded Jurkat cells

Figure 1 shows the typical fluorescence traces of Indo-1/AM-loaded Jurkat cells stimulated with monoclonal antibody to the T cell receptor (mAb C305; 1/10<sup>4</sup> dilution) or PHA (20  $\mu$ g ml<sup>-1</sup>). As has been shown by many investigators, these agonists cause a rapid and sustained rise in intracellular  $[Ca^{2+}]_i$ , which consists of both release of Ca<sup>2+</sup> from intracellular stores and transmembrane Ca<sup>2+</sup> influx (Tsien *et al.*, 1982; Nisbet-Brown *et al.*, 1985; Rabinovitch *et al.*, 1986; Gardner, 1989). In a similar fashion, stimulation by carbachol (CCh; 150  $\mu$ M) of Jurkat cells stably transfected with the human muscarinic receptor 1 (JHM1 cells) also generated a rapid and sustained rise in  $[Ca^{2+}]_i$  (Figure 1b). In each case of agonist stimulation, addition of 30  $\mu$ M SK&F 96365 caused a rapid reduction in the sustained phase of increase intracellular  $[Ca^{2+}]_i$  to a level approaching but greater than baseline  $[Ca^{2+}]_i$  (Figure 1a,b,c). When external Ca<sup>2+</sup> is removed by use of the Ca<sup>2+</sup> chelator EGTA (3 mM) prior to stimulation, a submaximal and transient rise in  $[Ca^{2+}]_i$  is seen, representing release from intracellular stores (shown for PHA in Figure 1d). SK&F 96365 has little to no effect on the agonist generated Ca<sup>2+</sup> signals in Ca<sup>2+</sup> free media (Figure 1d). Furthermore agonist-generated Ca<sup>2+</sup> responses after preincubation with SK&F 96365 are roughly equivalent to



**Figure 1** Sustained elevation of  $[\text{Ca}^{2+}]_i$  following receptor stimulation in Jurkat cells is inhibited by SK&F 96365. (a)  $[\text{Ca}^{2+}]_i$  measured by Indo-1 fluorescence, as a function of time, in a population of Jurkat cells after addition of mAb C305 ( $1/10^4$  dilution) to the T cell receptor, with or without addition of SK&F 96365 ( $30 \mu\text{M}$ ), to the extracellular solution. (b)  $[\text{Ca}^{2+}]_i$  in a population of JHM1 cells after addition of carbachol (CCh,  $150 \mu\text{M}$ ), with or without addition of SK&F 96365 ( $30 \mu\text{M}$ ), to extracellular solution. (c)  $[\text{Ca}^{2+}]_i$  in a population of Jurkat cells after addition of phytohaemagglutinin (PHA) with or without SK&F 9636 ( $30 \mu\text{M}$ ), to the extracellular solution. (d) Comparison of  $[\text{Ca}^{2+}]_i$  in two population of Jurkat cells, both of which were suspended in extracellular solution followed by chelation of external  $\text{Ca}^{2+}$  by EGTA ( $3 \text{ mM}$ ) as indicated by the arrow.

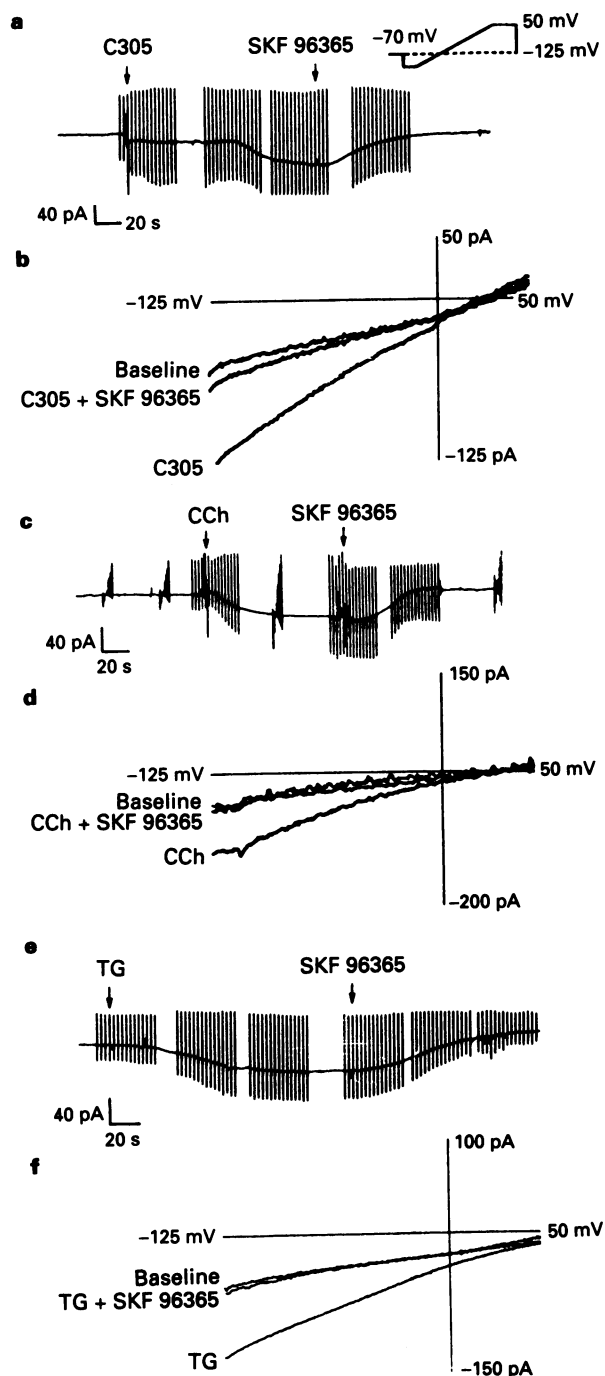
the responses seen after depletion of the extracellular  $\text{Ca}^{2+}$  pool by EGTA (data not shown). These results suggest that SK&F 96365 does not affect the release of  $\text{Ca}^{2+}$  from intracellular stores but rather selectively inhibits transmembrane  $\text{Ca}^{2+}$  influx. This agrees with the original observations on the effect of SK&F 96365 on agonist-stimulated  $[\text{Ca}^{2+}]_i$  responses in quin2-loaded human platelets (Graier *et al.*, 1992). The effect of SK&F 96365 was not due to general cell toxicity, since at all concentrations tested, the viability of cells exposed to the SK&F 96365, as checked by trypan blue exclusion, remained unaltered over a period of 48 h.

#### Effect of SK&F 96365 on whole-cell $\text{Ca}^{2+}$ current

Changes in  $[\text{Ca}^{2+}]_i$  measured by Indo-1/AM fluorescence represent indirect measurements of transmembrane  $\text{Ca}^{2+}$  entry, in aggregate with  $\text{Ca}^{2+}$  stores release,  $\text{Ca}^{2+}$  stores uptake, and transmembrane  $\text{Ca}^{2+}$  efflux. A direct assessment of the  $\text{Ca}^{2+}$  current underlying receptor-mediated  $\text{Ca}^{2+}$  influx can be attained by the whole cell patch clamp technique. By use of previously described protocols (McDonald *et al.*, 1993), we next tested the effect of SK&F 96365 on whole-cell  $\text{Ca}^{2+}$  currents after agonist stimulation. We and others have previously shown that stimulation of Jurkat cells by phosphoinositide-linked agonists activates whole-cell  $\text{Ca}^{2+}$  current (Lewis & Cahalan, 1989; McDonald *et al.*, 1993). The current is characterized by an inwardly rectifying  $I-V$  relation which approaches zero current asymptotically at very positive

potentials and is carried by a channel selective for divalent cations. In addition TG, a microsomal  $\text{Ca}^{2+}$ -ATPase inhibitor, activates  $\text{Ca}^{2+}$  current in T cells by depletion of intracellular  $\text{Ca}^{2+}$ -stores (Zweifach & Lewis, 1993; Premack *et al.*, 1994). The TG-induced  $\text{Ca}^{2+}$  current is indistinguishable from that activated by phosphoinositide-linked agonists (Premack *et al.*, 1994). By use of ionic solutions that maximize  $\text{Ca}^{2+}$  currents relative to other ionic species, we recorded the activation of  $\text{Ca}^{2+}$  current after stimulation of Jurkat cells with mAb C305 ( $1/10^4$  dilution) (Figure 2a), after stimulation of JHM1 cells with  $150 \mu\text{M}$  CCh (Figure 2c) or after stimulation of Jurkat cells with thapsigargin ( $100 \text{ nM}$ ) (Figure 2e). Baseline current measured from step protocols prior to stimulation was  $-0.49 \pm 0.3 \text{ pA/pF}$  (mean  $\pm$  s.d.) at  $-125 \text{ mV}$ ,  $n = 40$ . After stimulation, the peak current was  $-1.3 \pm 0.4 \text{ pA/pF}$  at  $-125 \text{ mV}$ ,  $n = 40$ . In each case the current was characterized by inward rectification and lack of measurable reversal over the voltage range studied (Figure 2b, 2d and 2f). Bath perfusion of SK&F 96365 ( $30 \mu\text{M}$ ) decreased ( $-0.5 \pm 0.2 \text{ pA/pF}$ , mean  $\pm$  s.d., at  $-125 \text{ mV}$ ,  $n = 30$ ) the mAb C305-induced  $\text{Ca}^{2+}$  current to approximately baseline over a 30 to 60 s interval (Figure 2a and 2b). Similarly  $30 \mu\text{M}$  SK&F 96365 inhibited CCh-induced  $\text{Ca}^{2+}$  current in JHM1 cells (Figure 2c and 2d) and TG-induced  $\text{Ca}^{2+}$  current in Jurkat cells (Figure 2e and 2f) to nearly baseline levels over a similar time course. The similarity of the effect of SK&F 96365 on the different modes current activation indicated that it is a functional antagonist, as was previously found in

other cell types (Merritt *et al.*, 1990). The effect of SK&F 96365 was reversible upon removal of the drug (data not shown) and dose-dependent. The relation between



**Figure 2** SK&F 96365 inhibits the inward  $\text{Ca}^{2+}$  current stimulated by mAb C305 in Jurkat cells, by carbachol (CCh) in JHM1 cells, or thapsigargin (TG) in Jurkat cells. (a) Continuous whole cell patch clamp current recording of a Jurkat cell during repetitive voltage clamp ramps from  $-125$  mV to  $+50$  mV from a holding potential of  $-70$  mV (protocol shown in insert). (b) Whole cell ramp current is plotted as a function of membrane potential (ramp  $I-V$ ) from the same cell as in (a), at baseline, after stimulation with mAb C305, and after subsequent perfusion with SK&F 96365. (c) Effect of CCh and SK&F 96365 on continuous whole cell patch clamp current recording of a JHM1 cell during voltage clamp ramps. (d) Ramp  $I-V$  from the same cell as in (c) at baseline after stimulation by CCh, and after subsequent perfusion with SK&F 96365. (e) Effect of TG and SK&F 96365 on continuous whole cell patch clamp current recording of a Jurkat cell during voltage clamp ramps; (f) ramp  $I-V$  from the same cell as in (e) at baseline, after stimulation by TG, and after subsequent perfusion with SK&F 96365.

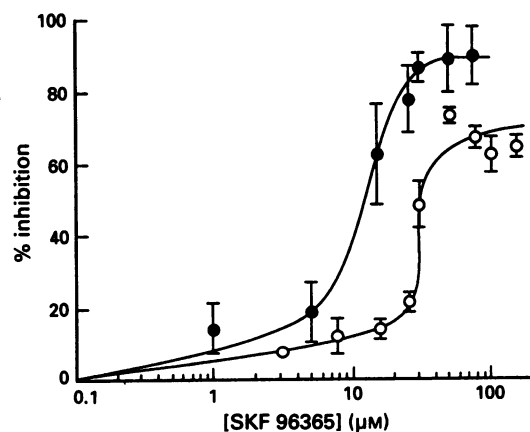
SK&F 96365 concentration and inhibition of  $\text{Ca}^{2+}$  influx as assessed by both Indo-1/AM fluorescence and whole-cell patch clamp recordings is shown in Figure 3. The  $\text{IC}_{50}$  for inhibition lies between  $12 \mu\text{M}$  (assessed whole-cell patch clamp) and  $20 \mu\text{M}$  (as assessed by Indo-1 fluorescence), while the maximal effect is seen at drug concentration greater than  $30 \mu\text{M}$ .

For purpose of comparison, the standard L-type voltage-gated  $\text{Ca}^{2+}$  channel blockers, nifedipine and diltiazem, were assessed for effect on the  $[\text{Ca}^{2+}]_i$  responses in Indo-1/AM loaded Jurkat cells. Neither nifedipine (up to  $50 \mu\text{M}$ ) nor diltiazem (up to  $1 \text{ mM}$ ) affected the fluorescence rise stimulated by mAb C305, PHA, or CCh (data not shown). Higher concentrations of nifedipine could not be tested due to autofluorescence of the drug at concentrations greater than  $50 \mu\text{M}$ . Whole-cell patch clamp recordings confirm that standard  $\text{Ca}^{2+}$  channel blockers do not inhibit the T cell  $\text{Ca}^{2+}$  current. Figure 4a shows a representative family of current traces obtained by voltage steps from a holding potential of  $-70$  mV at baseline, after stimulation by CCh, and after addition of SK&F 96365. As expected, the agonist-activated  $\text{Ca}^{2+}$  current showed little to no voltage- or time-dependent gating, differentiating the T cell  $\text{Ca}^{2+}$  channel from voltage-gated  $\text{Ca}^{2+}$  channels. SK&F 96365 blocks the current to nearly baseline levels. By contrast, high concentrations of diltiazem have no effect on the CCh-activated  $\text{Ca}^{2+}$  current in transfected Jurkat cells (Figure 4b), confirming the results derived from fluorescence studies.

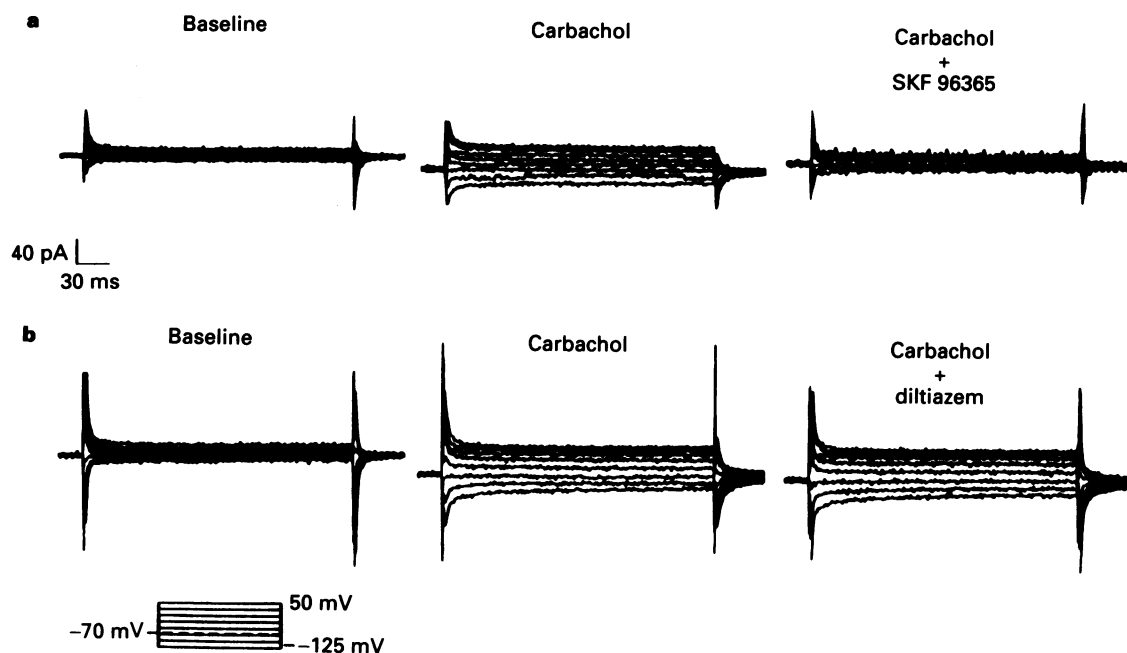
The ability of SK&F 96365 to inhibit  $\text{Ca}^{2+}$  current is unaffected by changes in the membrane holding potential. When the holding potential was varied over a range from  $-70$  mV to  $+25$  mV, no change is seen in the ability of SK&F 96365 to block  $\text{Ca}^{2+}$  current (at  $-70$  mV, 87% block,  $n = 10$ ; at  $-25$  mV, 92% block,  $n = 5$ ; at  $0$  mV, 98% block,  $n = 5$ ; at  $+25$  mV, 91% block,  $n = 4$ ). Both the magnitude of the current inhibition and its time course of inactivation kinetics remained the same as the membrane holding potential is made more positive.

#### Lack of effect of SK&F 96365 on whole-cell $\text{K}^+$ currents

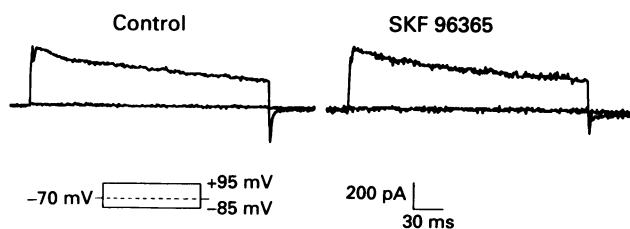
In order to rule out an indirect  $\text{Ca}^{2+}$  channel blockade via inhibition of  $\text{K}^+$  channels, the effect of SK&F 96365 on  $\text{K}^+$  currents was studied in Jurkat T cells. Outward  $\text{K}^+$  currents



**Figure 3** Dose-response curve for inhibition by SK&F 96365 of  $\text{Ca}^{2+}$  influx: (O)  $[\text{Ca}^{2+}]_i$  measured by Indo-1 fluorescence after stimulation of Jurkat cells by  $20 \mu\text{g ml}^{-1}$  phytohaemagglutinin (PHA), as a function of SK&F 96365 concentration; (●)  $\text{Ca}^{2+}$  current, as measured in single JHM1 cells by the whole-cell recording technique after stimulation by  $150 \mu\text{M}$  carbachol (CCh), as function of SK&F 96365 concentration. Inhibition is calculated as the percentage decrease from peak response relative to baseline. Data are expressed as mean  $\pm$  s.e. mean of five experiments (O), or seven cells (●). Where not shown, the error bars are within the symbol size.



**Figure 4** The voltage-gated Ca<sup>2+</sup> channel blocker diltiazem does not block the Ca<sup>2+</sup> current in Jurkat cells: (a) whole cell patch clamp record of steady-state currents during voltage steps (protocol demonstrated in the insert) from a JHM1 cell, at baseline, after stimulation by 150  $\mu$ M carbachol (CCh) and after addition of 30  $\mu$ M SK&F 96365. (b) Whole cell patch clamp records of steady-state currents during voltage steps from a JHM1 cell, at baseline, after stimulation by 150  $\mu$ M CCh, and after addition of 30  $\mu$ M diltiazem. Representative results of four experiments.



**Figure 5** SK&F 96365 does not block K<sup>+</sup> currents in Jurkat cells. Whole cell patch clamp record of K<sup>+</sup> current activated by depolarizing voltage steps (protocol shown in inset), at baseline (left) and after perfusion with 30  $\mu$ M SK&F 96365. Representative results of five experiments.

evoked by depolarizing voltage steps are demonstrated in Figure 5. Perfusion with 30  $\mu$ M SK&F 96365 had no apparent effect on either the magnitude of the K<sup>+</sup> current or its inactivation kinetics. Therefore, the action of SK&F 96365 in T cells is apparently specifically directed against agonist-generated Ca<sup>2+</sup> currents.

#### Effect of SK&F 96365 on cell growth and on IL-2 synthesis

Having shown that SK&F 96365 inhibits T cell Ca<sup>2+</sup> currents, we next used SK&F 96365 to see whether this inhibition was of functional importance. In order to assess the effect of SK&F 96365-mediated inhibition of Ca<sup>2+</sup> currents on mitogen-induced cell proliferation, peripheral blood lymphocytes were stimulated with PHA (20  $\mu$ g ml<sup>-1</sup>) or TG (100 nM) for 24 h, in the presence or absence of SK&F 96365. Subsequently cells were pulsed with [<sup>3</sup>H]-thymidine for an additional 24 h, harvested and incorporated radioactivity was determined. Surprisingly, TG alone induced cell proliferation, but the magnitude of proliferation was ten fold less than with PHA stimulation (Figure 6a). SK&F 96365 inhibi-

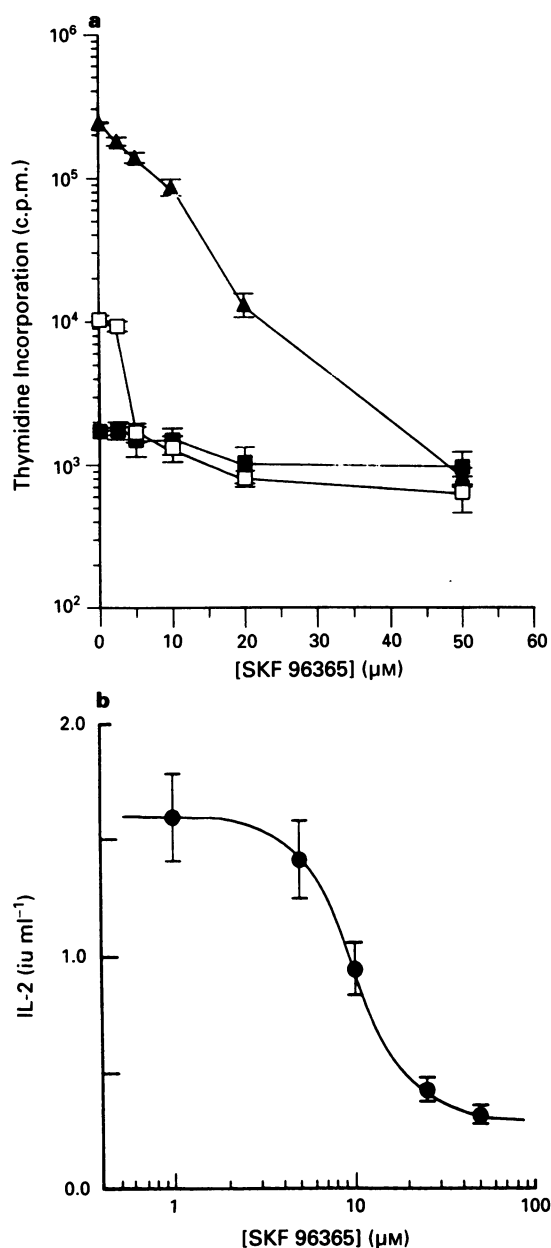
ted cell proliferation in a concentration-dependent fashion with an IC<sub>50</sub> of approximately 20  $\mu$ M for PHA-stimulated cells and 5  $\mu$ M for thapsigargin-stimulated cells. SK&F 96365 did not affect the viability of peripheral lymphocytes over a 48 h period as assessed by trypan blue exclusion.

IL-2, which expressed at the time T cells become committed to activation, is essential for T lymphocyte cell cycle progression, proliferation and immune function (Crabtree, 1989). Like T cell activation, the expression of IL-2 requires extracellular Ca<sup>2+</sup> (Mills *et al.*, 1985; Gelfand *et al.*, 1987). We next investigated whether SK&F 96365 inhibited the synthesis of IL-2. As demonstrated in Figure 6b, SK&F 96365 blocks IL-2 synthesis in a concentration-dependent fashion (IC<sub>50</sub> = 15  $\mu$ M) with a dose-response relationship close to that obtained for both inhibition of Ca<sup>2+</sup> current and inhibition of cell proliferation.

#### Discussion

Using fluorescence measurements of Indo-1 loaded cells and whole-cell patch clamp recording, we have shown that SK&F 96365 inhibits the *I<sub>crac</sub>* type Ca<sup>2+</sup> current stimulated in Jurkat T cells after depletion of Ca<sup>2+</sup> stores. IL-2 synthesis and [<sup>3</sup>H]-thymidine incorporation were inhibited in the similar concentration-range, indicating the functional significance of T cell Ca<sup>2+</sup> channel blockade. This concurs with previous findings in which it has been shown that SK&F 96365 inhibited proliferation of Jurkat cells by interfering with progression through the cell cycle (Nordstrom *et al.*, 1992).

Several early patch clamp studies of human peripheral T lymphocytes revealed that the predominant ion channels present are voltage-gated K<sup>+</sup> channels which closely resemble the delayed rectifier K<sup>+</sup> channels of nerve and muscle (DeCoursey *et al.*, 1984; Matteson & Deutsch, 1984; Deutsch *et al.*, 1986; Grissmer *et al.*, 1992). The lymphocyte K<sup>+</sup> current is blocked not only by K<sup>+</sup> channel blockers, but also by classical Ca<sup>2+</sup> channel antagonists such as verapamil



**Figure 6** Dose-response curves for inhibition by SK&F 96365 of T cell proliferation and IL-2 synthesis. (a) PBL's were cultured as described with 20  $\mu\text{g ml}^{-1}$  phytohaemagglutinin ( $\blacktriangle$ ) or 100 nM thapsigargin ( $\square$ ) plus SK&F 96365 at the indicated concentrations for 24 h. Each well was pulsed with [ $^3\text{H}$ ]-thymidine (1  $\mu\text{Ci}$ ) for 24 h. For control experiment, PBL was treated with SK&F 96365 alone ( $\blacksquare$ ). Data are shown as c.p.m.  $\pm$  s.e.mean of four samples. (b) Dose-response curve for inhibition by SK&F 96365 of the production of IL-2 activity in culture supernatants. Supernatants from PBL's stimulated with PHA (20  $\mu\text{g ml}^{-1}$ ) for 24 h were assayed on  $5 \times 10^3$  HT-2 cells/well. Data are shown as mean IL-2 activity  $\pm$  s.e.mean as determined by a standardized curve generated with recombinant IL-2, from triplicate samples.

( $\text{IC}_{50} = 6-7 \mu\text{M}$ ) and diltiazem ( $\text{IC}_{50} = 60 \mu\text{M}$ ) (Chandy *et al.*, 1984). The voltage-gated  $\text{K}^+$  channel is felt to play a role in maintaining  $\text{Ca}^{2+}$  influx during T cell activation. By repolarizing the cell toward the Nernstian equilibrium potential for  $\text{K}^+$  (about  $-80 \text{ mV}$ ), voltage-gated  $\text{K}^+$  channels may maintain the driving force for  $\text{Ca}^{2+}$ . Thus blockade of  $\text{K}^+$  channels may indirectly affect the rise in  $\text{Ca}^{2+}$  associated with agonist stimulation. At concentrations inhibitory to the  $\text{Ca}^{2+}$  current, no effect of SK&F 96365 on internal  $\text{Ca}^{2+}$  stores release or  $\text{K}^+$  currents were seen.

In a process comparable to that in T cells, receptor activation in other nonexcitable cell types results in an initial release of internal  $\text{Ca}^{2+}$  stores followed by sustained  $\text{Ca}^{2+}$  entry across the plasma membrane. Receptor-mediated  $\text{Ca}^{2+}$  entry (RCME) is mimicked by  $\text{Ca}^{2+}$  influx initiated by microsomal ATPase inhibitors, such as TG. These experimental findings provide strong support for the 'capacitative  $\text{Ca}^{2+}$  entry' model (Putney, 1990), in which emptying of intracellular  $\text{Ca}^{2+}$  stores activates  $\text{Ca}^{2+}$  influx across the plasma membrane by an unknown mechanism. RCME is often measured indirectly by monitoring  $\text{Ca}^{2+}$  or  $\text{Mn}^{2+}$  entry into cells loaded with fluorescent  $\text{Ca}^{2+}$  indicator dyes. By use of such techniques, it has been shown that a novel inhibitor, SK&F 96365, which is structurally distinct from known  $\text{Ca}^{2+}$  channel antagonists, inhibits RCME in platelets, neutrophils and endothelial cells (Merritt *et al.*, 1990; Mason *et al.*, 1991; Graier *et al.*, 1992). The  $\text{IC}_{50}$  for inhibition of RCME ranged from 8–12  $\mu\text{M}$ . SK&F 96365 showed selectivity in inhibiting RCME compared with internal stores release or  $\text{Ca}^{2+}$  currents through purinoceptors or NMDA receptors, but not with voltage-gated  $\text{Ca}^{2+}$  currents (Merritt *et al.*, 1990). Similar concentrations of SK&F 96365 also inhibited platelet aggregation, adhesion, and chemotaxis of neutrophils (Merritt *et al.*, 1990) and the synthesis and release of NO in endothelial cells (Graier *et al.*, 1992), substantiating the functional importance of RCME blockade in these cell types.

Recently,  $I_{\text{crac}}$ , the  $\text{Ca}^{2+}$  current activated by depletion of internal  $\text{Ca}^{2+}$  stores, was described in mast cells (Hoth & Penner, 1992). A  $\text{Ca}^{2+}$  current with nearly identical properties is also seen in T cells after internal generation of  $\text{InsP}_3$  (McDonald *et al.*, 1993) or after stores depletion by thapsigargin (Zweifach & Lewis, 1993; Premack *et al.*, 1994). The  $I_{\text{crac}}$  class of  $\text{Ca}^{2+}$  currents appears to be partially or wholly responsible for RCME (Clapham, 1993). Our finding that SK&F 96365 inhibits this current in Jurkat T cells is the first report of an organic antagonist for  $I_{\text{crac}}$ .

The latency with which SK&F 96365 produces full blockade of Jurkat T cell  $\text{Ca}^{2+}$  current (up to 60 s) raises the question of whether SK&F 96365 works directly through an association with the  $\text{Ca}^{2+}$  channel versus indirectly by interfering with intermediate biochemical steps. The mechanism by which depletion of  $\text{Ca}^{2+}$  stores signals activation of  $I_{\text{crac}}$  is largely unknown (Clapham, 1993), though recent studies suggest the synthesis of a novel, diffusible, phosphorylated second messenger (Parekh *et al.*, 1993; Randriamampita & Tsien, 1993). Suggestions that SK&F 96365 may be working by indirect mechanisms involving the intermediate biosynthetic pathway of a requisite second messenger have been made on the basis that this drug closely resembles the imidazole antimycotics, econazole, miconazole, and clotrimazole, which are thought to be selective antagonists of cytochrome P-450. Importantly the cytochrome P-450 inhibitors have also been noted to inhibit  $\text{Mn}^{2+}$  entry in rat thymocytes and human neutrophils, raising the possibility that cytochrome P-450 is involved in regulating plasma membrane  $\text{Ca}^{2+}$  permeability (Alonso *et al.*, 1989; Alvarez *et al.*, 1992; Montero *et al.*, 1991, 1993; Sargeant *et al.*, 1992). In the hepatic microsome, cytochrome P-450 directly inhibits the activity of the endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase pump through an oxygen-dependent oxidative process, further substantiating a role for this enzyme in  $\text{Ca}^{2+}$  homeostasis. Two cytochromes (P1-450 or P448) have been found in microsomes of human lymphocytes after mitogen stimulation, where they are responsible for enzymatic activities such as aryl hydrocarbon hydroxylase (Amsbaugh *et al.*, 1986).

Additional suggestion that SK&F 96365 may inhibit  $\text{Ca}^{2+}$  channels by indirect means is provided by the observation that this drug interacts with the  $\text{Ca}^{2+}$  binding regulatory protein calmodulin as measured by  $^1\text{H}$ -nuclear magnetic resonance (n.m.r.) (Reid *et al.*, 1990). Since calmodulin antagonists have been shown to facilitate RCME (Rao, 1987; Alvarez *et al.*, 1992), it remains possible that SK&F blocks  $I_{\text{crac}}$  through a calmodulin-related action. Histochemical

studies have shown that the endoplasmic reticulum contains significant amounts of calmodulin (Nordstrom *et al.*, 1992). A role for calmodulin in modulating Ca<sup>2+</sup> influx is suggested by the finding that several calmodulin antagonists (phenothiazine, trifluoperazine, fluphenazine and chlorpromazine) increase the uptake of Mn<sup>2+</sup> by thymocytes whose Ca<sup>2+</sup> stores are filled (Alonso *et al.*, 1989; Schleuning *et al.*, 1989).

In conclusion, we present evidence that SK&F 96365 inhibits Ca<sup>2+</sup> current in T cells and is thus the first reported organic inhibitor of I<sub>crac</sub> type Ca<sup>2+</sup> channels. The mechanism by which this drug inhibits I<sub>crac</sub> is largely unknown, but may involve either direct channel blockade versus interference with a requisite intermediate biochemical step. The low potency (IC<sub>50</sub> = 12 μM), lack of selectivity (also blocks voltage-gated Ca<sup>2+</sup> channels; Merritt *et al.*, 1990) and lack of

knowledge about the precise mechanism by which it works limit this drug to the status of an experimental tool. The functional consequence of Ca<sup>2+</sup> channel blockade in T cells, however, further emphasizes the potential therapeutic value of higher potency, more selective I<sub>crac</sub> blockers.

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## References

- ALONSO, M.T., SANCHEZ, A. & GARCIA-SANCHO, J. (1989). Monitoring of the activation of receptor-operated Ca<sup>2+</sup> channel in human platelets. *Biochem. Biophys. Res. Commun.*, **162**, 24–29.
- ALVAREZ, J., MONTERO, M. & GARCIA-SANCHO, J. (1992). Cytochrome P450 may regulate plasma membrane Ca<sup>2+</sup> permeability according to filing states of the intracellular Ca<sup>2+</sup> stores. *FASEB J.*, **6**, 786–792.
- AMSBAGH, S.C., DING, J., SWAN, D.C., POPESCU, N.C. & CHEN, Y. (1986). Expression and Chromosomal localization of the cytochrome P1-450 gene in human mitogen-stimulated lymphocytes. *Cancer Res.*, **46**, 2423–2427.
- BROWN, A.M., KUNZE, D.L. & YATANI, A. (1984). The agonist effect of dihydropyridines on Ca<sup>2+</sup> channels. *Nature*, **311**, 570–572.
- CHANDY, K.G., DECOURSEY, T.E., CAHALAN, M.D., MCLAUGHLIN, C. & GUPTA, S. (1984). Voltage-gated K<sup>+</sup> channels are required for human T lymphocyte activation. *J. Exp. Med.*, **160**, 369–385.
- CLAPHAM, D. (1993). A mysterious new influx factor. *Nature*, **364**, 763–764.
- CRABTREE, G. (1989). Contingent genetic regulatory events in T lymphocyte activation. *Science*, **243**, 355–361.
- DECOURSEY, T.E., CHANDY, K.G., GUPTA, S. & CAHALAN, M.D. (1984). Voltage-gated K<sup>+</sup> channels in human T lymphocytes: a role in mitogenesis? *Nature*, **307**, 465–468.
- DEUTSCH, C., KRAUSE, D. & LEE, S.C. (1986). Voltage-gated K<sup>+</sup> conductance in human T lymphocytes stimulated with phorbol ester. *J. Physiol.*, **372**, 405–423.
- GARDNER, P. (1989). Ca<sup>2+</sup> and T lymphocyte activation. *Cell*, **59**, 15–20.
- GELFAND, E.W., MILLS, G.B., CHEUNG, R.K., LEE, L.W. & GRINSTEIN, S. (1987). Transmembrane ion fluxes during activation of human T lymphocytes: Role of Ca<sup>2+</sup>, Na<sup>+</sup>/H<sup>+</sup> Exchange and phospholipid turnover. *Immunol. Rev.*, **95**, 59–87.
- GOLDSMITH, M.A., DESAI, D.M., SCHULTZ, T. & WEISS, A. (1989). Function of a heterologous muscarinic receptor in T cell antigen receptor signal transduction mutants. *J. Biol. Chem.*, **264**, 17190–17197.
- GRAIER, W.F., GROSCHNER, K., SCHMIDT, K. & KUKOVETZ, W.R. (1992). SK&F 96365 inhibits histamine induced formation of endothelium-derived relaxing factor in human endothelial cells. *Biochem. Biophys. Res. Commun.*, **186**, 1539–1545.
- GRISSMER, S., LEWIS, R.S. & CAHALAN, M.D. (1992). Ca<sup>2+</sup> activated K<sup>+</sup> channel in human leukemic T cell. *J. Gen. Physiol.*, **99**, 63–84.
- GRYNKEWIZ, G., POENIC, M. & TSIEN, R.Y. (1985). A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3448.
- HAMIL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.
- HESS, P. & TSIEN, R.W. (1984). Different modes of Ca<sup>2+</sup> channel gating behavior favored by dihydropyridine Ca<sup>2+</sup> agonist and antagonist. *Nature*, **311**, 538–544.
- HOTH, M. & PENNER, R. (1992). Depletion of Intracellular Ca<sup>2+</sup> stores activates a Ca<sup>2+</sup> current in mast cells. *Nature*, **355**, 353–356.
- JY, W., FREIGIEN, N., BOURGUIGNON, G.J. & BOURGUIGNON, L.Y.W. (1989). Role of Ca<sup>2+</sup> during lymphocyte activation. *Biochem. Biophys. Acta.*, **983**, 153–160.
- KUNO, M., GORONZY, J., WEYAND, D. & GARDNER, P. (1986). Single-channel and whole cell recordings of mitogen-regulated inward currents in human cloned helper T lymphocytes. *Nature*, **323**, 269–273.
- KUNO, M. & GARDNER, P. (1987). Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T lymphocytes. *Nature*, **326**, 301–304.
- LEE, K.S. & TSIEN, R.W. (1983). Mechanism of Ca<sup>2+</sup> channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialyzed heart cells. *Nature*, **302**, 790–794.
- LEWIS, R.S. & CAHALAN, M.D. (1989). Mitogen-induced oscillations of cytosolic Ca<sup>2+</sup> and transmembrane Ca<sup>2+</sup> current in human leukemic T cells. *Cell Regulation*, **1**, 99–112.
- MASON, M.J., MAHAUT-SMITH, M.P. & GRINSTEIN, S. (1991). The role of intracellular Ca<sup>2+</sup> in the regulation of the plasma membrane Ca<sup>2+</sup> permeability of unstimulated rat lymphocytes. *J. Biol. Chem.*, **266**, 10872–10879.
- MATESON, D.R. & DEUTSCH, C. (1984). K<sup>+</sup> channels in T lymphocytes: a patch clamp study using monoclonal antibody adhesion. *Nature*, **307**, 468–471.
- MCDONALD, T.V., PREMACK, B.A. & GARDNER, P. (1993). Flash photolysis of caged inositol 1,4,5-trisphosphate activates plasma membrane Ca<sup>2+</sup> current in human T cells. *J. Biol. Chem.*, **268**, 3889–3896.
- MERRITT, J.E., ARMSTRONG, W.P., BENHAM, C.D., HALLAM, T.J., JACOB, R., JAXACHAMIEC, A., LEIGH, B.K., MCCARTHY, S.A., MOORES, K.E. & RINK, T.J. (1990). SK&F 96365, a novel inhibitor of receptor-mediated Ca<sup>2+</sup> entry. *Biochem. J.*, **271**, 515–522.
- MIGNERY, G.A., JOHNSTON, P.A. & SUDHOF, T.C. (1992). Mechanism of Ca<sup>2+</sup> inhibition of inositol 1,4,5-trisphosphate (IP3) binding to the cellular IP3 receptor. *J. Biol. Chem.*, **267**, 7450–7455.
- MILLS, G.B., CHEUNG, R.K., CRAGOE, E., GRINSTEIN, S. & GELFAND, E.W. (1985). Increase in cytosolic free Ca<sup>2+</sup> concentration is an intracellular messenger for production of IL-2. *J. Immunol.*, **134**, 1640–1643.
- MONTERO, M., ALVAREZ, J. & GARCIA-SANCHO, J. (1991). Agonist-induced Ca<sup>2+</sup> influx in human neutrophils is secondary to the emptying of intracellular Ca<sup>2+</sup> stores. *Biochem. J.*, **277**, 73–79.
- MONTERO, M., GARCIA-SANCHO, J. & ALVAREZ, J. (1993). Comparative effects of cytochrome P450 inhibitors of Ca<sup>2+</sup> and Mn<sup>2+</sup> entry. *Biochim. Biophys. Acta.*, **1177**, 127–133.
- NISBET-BROWN, E., CHEUNG, R.K., LEE, J. & GELFAND, E.W. (1985). Antigen-dependent increase in cytosolic Ca<sup>2+</sup> in human T lymphocyte. *Nature*, **316**, 545–547.
- NORDSTROM, T., NEVANLINNA, H.A. & ANDERSSON, L.C. (1992). Mitosis-arresting effect of the Ca<sup>2+</sup> channel inhibitor SK&F 96365 on human leukemia cells. *Exp. Cell Res.*, **202**, 487–494.
- NOWYCKY, M.C., FOX, A.P. & TSIEN, R.W. (1985). Three types of neuronal Ca<sup>2+</sup> channel with different Ca<sup>2+</sup> agonist sensitivity. *Nature*, **316**, 440–443.
- PAREKH, A.G., TERLAU, H. & STUHMER, W. (1993). Depletion of IP3 stores activates a Ca<sup>2+</sup> and K<sup>+</sup> current by means of a phosphatase and a diffusible messenger. *Nature*, **364**, 814–818.
- PREMACK, B.A., MCDONALD, T.V. & GARDNER, P. (1994). Activation of Ca<sup>2+</sup> currents in Jurkat T cells following the Depletion of Ca<sup>2+</sup> stores by microsomal Ca<sup>2+</sup>-ATPase Inhibitor. *J. Immunol.*, **15**, 5226–5240.

- PUTNEY, J.W. Jr. (1990). Capacitative  $\text{Ca}^{2+}$  entry revisited. *Cell Calcium*, **11**, 611–624.
- RABINOVITCH, P., JUNE, C., GROSSMANN, A. & LEDBETTER, J. (1986). Heterogeneity among T cells in intracellular free  $\text{Ca}^{2+}$  responses after mitogen stimulation with PHA or anti-CD3. *J. Immunol.*, **137**, 952–961.
- RANDRIAMAMPITA, C. & TSIEN, R.Y. (1993). Emptying of intracellular  $\text{Ca}^{2+}$  stores releases a novel small messenger that stimulates  $\text{Ca}^{2+}$  influx. *Nature*, **364**, 809–814.
- RAO, G.H. (1987). Influence of calmodulin antagonist on agonist induce  $\text{Ca}^{2+}$  mobilization and platelet activation. *Biochem. Biophys. Res. Commun.*, **148**, 768–775.
- REID, D.G., MACLACHLAN, L.K., ROBINSON, S.P., CAMILLERI, P., DYKE, C.A. & THORPE, C.J. (1990). Calmodulin discriminates between the two enantiomer of the receptor-operated  $\text{Ca}^{2+}$  channel blocker SK&F 96365. *Chirality*, **2**, 229–232.
- SARGEANT, P., CLARKSON, W.D., SAGE, S.O. & HEEMSKERK, J.W. (1992).  $\text{Ca}^{2+}$  influx evoked by  $\text{Ca}^{2+}$  store depletion in human platelets is more susceptible to cytochrome P450 than receptor mediated  $\text{Ca}^{2+}$  entry. *Cell Calcium*, **13**, 553–564.
- SCHLEUNING, M.J., DUGGAN, A. & REEM, G.H. (1989). Inhibition by chlorpromazine of lymphokine-specific mRNA expression in human thymocytes. *Eur. J. Immunol.*, **19**, 1491–1496.
- TSIEN, R.W., ELLINOR, P.T. & HOME, W.A. (1991). Molecular Diversity of voltage-dependent  $\text{Ca}^{2+}$  channels. *Trends Pharmacol. Sci.*, **12**, 349–354.
- TSIEN, R.Y., POSSAN, T. & RINK, T.J. (1982). T cell mitogens cause early changes in cytoplasmic-free  $\text{Ca}^{2+}$  and membrane potential in lymphocytes. *Nature*, **295**, 68–71.
- ZWEIFACH, A. & LEWIS, R.S. (1993). Mitogen-regulated  $\text{Ca}^{2+}$  current of T lymphocytes is activated by depletion of intracellular  $\text{Ca}^{2+}$  stores. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 6295–6299.

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