RESEARCH COMMUNICATION

Primaquine, an inhibitor of vesicular transport, blocks the calcium-release-activated current in rat megakaryocytes

Baggi SOMASUNDARAM,* Jim C. NORMAN and Martyn P. MAHAUT-SMITH

The Physiological Laboratory, University of Cambridge, Downing Street, Cambridge, CB2 3EG, U.K.

The whole-cell patch-clamp technique was used to study the effect of primaquine, an inhibitor of vesicular transport, on the calcium-release-activated current (I_{crac}) in rat megakaryocytes. Addition of primaquine, before emptying of internal Ca^{2+} stores by ionomycin, prevented the development of I_{crac} , with a half-maximal concentration of near 100 μM . Maximal inhibition ($\geq 83\%$) was observed at 0.6–1 mM primaquine. At 1 mM, chloroquine, a related compound which is less effective at blocking vesicular secretion, had no effect on I_{crac} . Primaquine (0.8 mM) added after sustained activation of I_{crac} caused a gradual block of

current, with maximal inhibition of 50% observed after 2–3 min. At 1 mM, internal guanosine 5'-[γ -thio]triphosphate reduced I_{crac} by $65 \pm 13\%$. Neither 1 mM GTP nor 2 mM guanosine 5'-[β -thio]diphosphate had any significant effect on I_{crac} . The recognized role of GTPases in the regulation of vesicular trafficking, together with block of I_{crac} activation by primaquine, provide evidence that the channels carrying I_{crac} may be stored in a vesicular membrane compartment and transferred to the plasma membrane following store depletion.

INTRODUCTION

A Ca^{2+} current gated by release of Ca^{2+} from InsP_3 -dependent intracellular stores has recently been measured in a variety of cells and termed 'calcium-release-activated current' (I_{crac}) [1–3]. The actual mechanism whereby a reduction of Ca^{2+} content in the stores is coupled to the activation of I_{crac} has been the topic of intense recent investigation. A direct physical link between store and plasma membranes has been postulated [4,5], although more evidence has accumulated recently in favour of a mobile second messenger, released from the stores following Ca^{2+} depletion, which can then diffuse to the plasma membrane and activate a Ca^{2+} influx [3]. Candidates for this messenger include a small GTPase [6,7] and calcium-influx factor ('CIF'), a novel messenger [8]. In addition, cytochrome *P*-450, a phosphatase, tyrosine phosphorylation and cyclic GMP have also been proposed to have roles in controlling the store-dependent Ca^{2+} entry pathway [9].

In whole-cell patch-clamp recordings, activation of I_{crac} by store depletion runs down with an exponential time course, implying the dialysis of a diffusible cytosolic factor [7]. The predicted size of the labile factor, together with evidence for a step requiring GTP hydrolysis in the activation of I_{crac} or store-dependent Ca^{2+} entry, suggest that the factor may be a small GTP-binding protein [6,7]. Interestingly, once activated, I_{crac} is sustained, and guanosine 5'-[γ -thio]triphosphate (GTP[S]) has no effect; therefore a GTP-dependent step is required only to recruit the Ca^{2+} conductance and not to maintain it. Small GTP-binding proteins, including members of the Rab and ARF families, have well recognized roles in the control of vesicle-mediated protein transport [10], and a number of channels and carrier proteins are known to undergo regulation by vesicular trafficking [11]. The anti-malarial amine primaquine has recently been shown to block protein secretion by inactivating the membranes that form transport vesicles, but not the membranes

that are the target of the vesicles [12]. In the present study we have used primaquine to investigate the hypothesis that calcium-release-activated channels are stored in vesicles under the plasma membrane and inserted into the membrane following store release [3,7].

METHODS

Adult male Wistar rats were killed by cervical dislocation. Megakaryocytes from the femoral and tibial marrow were prepared, and membrane currents were recorded using standard whole-cell patch-clamp techniques with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, U.S.A.) as previously described [2]. Cells were isolated and stored in a solution containing (mM) 140 NaCl, 5 KCl, 1 MgCl_2 , 2 CaCl_2 , 10 glucose and 10 Hepes, pH 7.4 (adjusted with Tris base). All experiments were conducted in a solution where Na^+ and K^+ ions were replaced by an equal concentration of *n*-methyl-D-glucamine (NMDG $^+$) and Cs^+ respectively. The internal saline contained (mM) 100 caesium gluconate, 10 Cs_4BAPTA [tetra-caesium bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetate], 2 CaCl_2 , 5 NaCl, 2 MgCl_2 , 10 Hepes, 0.2 Na_2GTP , pH 7.4 (adjusted with Tris base). This Ca^{2+} /BAPTA mixture maintained the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) near resting cytosolic levels and reduced the chance of spontaneous activation of I_{crac} due to leak of Ca^{2+} from internal stores that often occurred with BAPTA alone. Ionomycin and thapsigargin were applied from a puffer pipette placed 150 μm from the cell. A diffusible factor required for activation of I_{crac} was washed out during whole-cell recordings from rat megakaryocytes in a manner similar to that previously demonstrated for rat basophilic leukaemia (RBL) cells [7]. The rate of dialysis of the cytoplasm will depend upon the whole-cell access resistance; therefore, to allow comparison

Abbreviations used: I_{crac} , calcium-release-activated current; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; NMDG $^+$, *n*-methyl-D-glucamine; BAPTA, bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; RBL, rat basophilic leukaemia cells.

* To whom correspondence should be sent.

Figure 1 Effect of primaquine on activation of I_{crac}

(a) and (c) Continuous record of whole-cell membrane current at -10 mV. (b) and (d) Currents generated by a voltage ramp from -100 to $+70$ mV before and after $3 \mu\text{M}$ ionomycin (1 and 2 respectively). (a) and (b) Control cell; (c) and (d), cell exposed to 0.6 mM primaquine, added 5 min before ionomycin. Cell capacitances were 87 pF for the control and 100 pF for the primaquine-treated cell. I_{crac} was activated by a 5 s exposure to ionomycin at $9 \text{ s} \cdot \text{M}\Omega^{-1}$ after initial break-in to whole-cell configuration.

of I_{crac} in different cells, the time after break-through to whole-cell mode was normalized for series resistance, and ionomycin was applied at a standard normalized time range of $9\text{--}10 \text{ s} \cdot \text{M}\Omega^{-1}$ for primaquine and chloroquine experiments and a range of $5\text{--}15 \text{ s} \cdot \text{M}\Omega^{-1}$ for experiments with guanosine phosphates. Cells were held at -10 or -20 mV and current-voltage relationships obtained at regular intervals using 300 ms voltage ramps from -100 to $+60$ or $+70$ mV. Currents were normalized for cell capacitance and I_{crac} was measured as the amount of inward current that developed in response to ionomycin at -70 mV. This is close to the Cl^- reversal potential and therefore removed any contribution from Cl^- currents that developed in some cells. Some variability in the peak amplitude of I_{crac} was observed between preparations; therefore, wherever possible, results are expressed as a percentage of controls from the same batch of cells. Cs_4BAPTA was from Molecular Probes (Eugene, OR, U.S.A.). GTP, GDP and their analogues (GTP[S] and GDP[S]), and ionomycin and thapsigargin, were from Calbiochem. All other reagents were from Sigma. Experiments were carried out at room temperature ($20\text{--}24^\circ\text{C}$) within 24 h of cell isolation. Results are expressed as means $\pm$ S.D.

RESULTS AND DISCUSSION

To study store-dependent Ca^{2+} currents (I_{crac}) in rat megakaryocytes, we used conditions previously shown to amplify this small current and minimize contamination by other ionic currents ([1,2]; see the Methods section). Under whole-cell voltage clamp, following blockade of K^+ currents by Cs^+ in the pipette, $3 \mu\text{M}$

Figure 2 Dose-dependency of primaquine block; effect of chloroquine

Primaquine ($0.1\text{--}1 \text{ mM}$) or chloroquine (1 mM) were added to the bath before activation of ionomycin. Peak I_{crac} was measured at -70 mV and normalized for cell capacitance; $n = 5$ for each concentration. Results are expressed as percentage inhibition of the average control response.

ionomycin evoked an inward current at a holding potential of -10 mV (Figure 1a). Further applications of ionomycin shortly after the current peaked failed to elicit any additional inward current (result not shown), indicating that the Ca^{2+} stores were empty and I_{crac} was maximally activated. Figure 1(b) shows the currents generated by voltage ramps from -100 to $+70$ mV before ionomycin and after maximal development of inward current. The ionomycin-evoked inward current had a curvilinear current-voltage relationship and displayed little or no reversal

Figure 3 Effect of primaquine and Zn²⁺ on pre-activated *I*_{crac}

(a) Continuous record of membrane current at -20 mV. Ionomycin (Iono) at 3 μM was applied to empty the Ca²⁺ stores and maximally activated *I*_{crac}. Primaquine (1 mM) and Zn²⁺ (500 μM) were superfused throughout the time indicated by the bars. (b) Currents generated by voltage ramps from -100 to +60 mV at the three time points indicated during the experiment of (a). (c) Mean current density at -70 mV before and after activation of *I*_{crac} with a 5 s application of 1 μM thapsigargin and 3 μM ionomycin (arrow). (d) Effect of 0.8 mM primaquine on the mean current density (-70 mV) after activation of *I*_{crac} as in (c). In (c) and (d) the cells were held at -20 mV and the current measured from voltage ramp protocols (*n* = 5 in c and d; the bars represent S.E.M.).

within the voltage range studied (Figure 1b). We have previously demonstrated this inwardly rectifying current to be carried by a pathway highly selective for Ca²⁺ [2] and indistinguishable from *I*_{crac} in other cells [3].

The receptor-recycling inhibitor primaquine [13,14], added to the bath at a concentration of 0.6 mM, 5 min before emptying of internal stores, markedly reduced the maximal *I*_{crac} that developed in response to ionomycin (Figure 1c). As shown by the ramp-generated currents in Figure 1(d), obtained before and after ionomycin, primaquine reduced the store-dependent inward current throughout the voltage range of -100 to +70 mV. Block of *I*_{crac} by pre-exposure to primaquine was dose-dependent, with a half-maximal concentration of approx. 100 μM (Figure 2). Maximum inhibition (85 ± 1% of control) was observed at 0.6 mM primaquine. These are concentrations of primaquine previously found to block intracellular transport processes, including receptor recycling and protein secretion [12–14]. Chloroquine, a related compound which can also act as an inhibitor of receptor recycling [13,14], was ineffective as an inhibitor of *I*_{crac} in megakaryocytes at concentrations of up to 1 mM (Figure 1c). Therefore at least one site of primaquine block is unaffected by

chloroquine and involved in the activation mechanism for *I*_{crac}. An alternative explanation is that primaquine, but not chloroquine, directly inhibits the channels underlying *I*_{crac}. To test this, we first emptied the Ca²⁺ stores with ionomycin and then superfused primaquine to examine the effect on steady-state *I*_{crac}. In the experiment shown in Figure 3(a), 1 mM primaquine caused a gradual reduction of ionomycin-evoked current at the holding potential of -20 mV. This effect was not readily reversible. In the same cell, however, 500 μM Zn²⁺, which is a potent competitive antagonist of *I*_{crac} [15], rapidly and reversibly abolished the remaining store-dependent inward current. This smaller effect of primaquine compared with Zn²⁺ was consistent across the voltage range studied (-100 to 60 mV), as shown by the ramp currents in Figure 3(b), (1) after maximal activation of *I*_{crac}, (2) after primaquine and (3) in the presence of Zn²⁺. To compare the maximal effect of primaquine before and after activation of *I*_{crac}, longer post-activation exposures were required; however, in many cells *I*_{crac} tended to inactivate over time. To prevent store refilling from contributing to the process of inactivation, 1 μM thapsigargin was added to the injection saline. Following activation by ionomycin and thapsigargin, the

mean current, measured at -70 mV and averaged from five cells, showed only a small decline over a 10 min period (Figure 3c). At 0.8 mM, primaquine caused a gradual reduction in current, which was maximal within 2–3 min (Figure 3d). After 7 min exposure to primaquine, the mean current was reduced to 43 % of peak current in these cells, although the control at this time was also reduced, to 86 % of peak; thus primaquine caused a 50 % reduction in current compared with control cells. This compares with 83–85 % block of I_{crac} by 0.6 – 1 mM primaquine added before I_{crac} activation (see Figure 2). Together, these results suggest that primaquine has less effect on the I_{crac} once it has been stimulated by Ca^{2+} store depletion, and is more effective at blocking the activation mechanism of the current. Primaquine was not acting merely by preventing ionomycin-induced Ca^{2+} store depletion, since it did not block the activation of Ca^{2+} -dependent K^+ current by ionomycin recorded in Ca^{2+} -free external saline with potassium gluconate internal saline containing normal (0.1 mM EGTA) Ca^{2+} buffering (results not shown).

Primaquine and chloroquine have both been used to inhibit receptor recycling in a number of cell types [13,14]. This action has been attributed to their ability, as weak bases, to raise the pH of endosomes and thus interfere with enzyme processes within these compartments. Primaquine, however, has more extensive effects on vesicular transport than chloroquine [12–14,16]. It has been proposed that these effects reflect its ability to prevent formation of transport vesicles within the vesicular secretion pathway [12,16]. In a cell-free Golgi-transport assay, Hiebsch et al. [12] showed that, at concentrations (K_d approx. 50 μ M) similar to those found to block I_{crac} activation in the present study, primaquine inhibits the budding of vesicles without affecting the fusion of preformed vesicles to their target membrane. More than 50-fold higher levels of chloroquine are required to block transport within the reconstituted Golgi system. In our study, chloroquine was without effect on I_{crac} at up to 1 mM, the concentration at which primaquine blocked activation of the current by 83 %. Thus the channels underlying I_{crac} may be stored within an endo-membrane compartment and activated by inclusion into primaquine-sensitive budding vesicles that subsequently fuse with the plasma membrane. The smaller, slow block of I_{crac} observed when primaquine was applied following store depletion (Figure 3a) may be due to continuous retrieval, by endocytosis, of the channel from the plasma membrane. A small fall in current would therefore be expected if the cell were unable to replenish the plasma membrane pool of I_{crac} channel in the presence of primaquine. The present data suggest that the I_{crac} channels are sequestered in a compartment, such as the *trans*-Golgi network or early endosomes, through which there is a constant flux of lipid traffic both from and to the plasma membrane. Store depletion would therefore de-sequester the channel such that it could follow this pre-existing route to the plasma membrane and cycle freely between the compartment and the cell surface. Primaquine would be expected to reduce the amount of pre-activated I_{crac} channel by allowing endocytosis to proceed until such time as the plasma membrane runs out of lipid available for internalization. Previous studies have suggested that this point is reached after endocytosis of approx. 50 % of the plasma-membrane area, which is after about 2 min at 37°C with 0.6 mM primaquine [17]. This effect of primaquine can therefore account for the slowly developing reduction of pre-activated I_{crac} which amounted to 50 % block in 0.8 mM primaquine (Figure 3d). However, we cannot rule out the possibility that primaquine block of activated I_{crac} was in part the result of a slowly developing direct action on the I_{crac} channel once inserted into the plasma membrane.

Table 1 Effect of GTP[S], GTP and GDP[S] on I_{crac}

Peak I_{crac} currents evoked by 3 μ M ionomycin at -70 mV were measured in the absence of GTP (control) and with GTP[S] (1 mM), GTP (1 mM), or GDP[S] (2 mM) added to the pipette saline. Results are expressed as the mean current density (pA/pF) and as percentage block of the control. Only currents in the presence of GTP[S] were significantly different (* $P < 0.001$) from currents under control conditions.

Condition	Current density (pA/pF)	Block (%)	No. of expts.
Control	-1.00 ± 0.3	—	13
GTP[S]	$-0.35 \pm 0.18^*$	$65 \pm 7\%$	13
GTP	-0.80 ± 0.4	$20 \pm 16\%$	13
GDP[S]	-0.89 ± 0.2	$11 \pm 11\%$	6

The notion that store depletion causes insertion of I_{crac} channels into the plasma membrane from specialized vesicles has been considered previously [3]. A role for vesicular trafficking, suggested following patch-clamp studies in RBL cells, was based on evidence for a small GTPase in triggering I_{crac} and the established role of these proteins in regulating vesicular transport [6,7]. We were therefore interested in whether a GTP-dependent step was also involved in activation of I_{crac} in rat megakaryocytes. As the data in Table 1 shows, addition of 1 mM GTP[S], a non-hydrolysable analogue of GTP, to the pipette saline significantly reduced the amplitude of I_{crac} activated by ionomycin. If on the other hand, GTP (1 mM) or the non-hydrolysable analogue of GDP, GDP[S] (2 mM), were added to the internal saline, no significant effect on store-dependent currents was observed compared with the control. Similar results have been reported for RBL cells [7] and provide evidence for a step requiring GTP hydrolysis during the activation mechanism for I_{crac} . Vesicular transport has a requirement for GTP-hydrolysis (by the small GTPase, ARF) and is inhibited by very low (K_d approx. 10 μ M) concentrations of GTP[S] [18,19]. Much higher (K_d approx. 2 mM) levels of GDP[S] are required to block this kind of transport [19]. Heterotrimeric G-protein-regulated processes, on the other hand, are blocked by much lower (K_d approx. 0.4 – 2 μ M) concentrations of GDP[S] [20]. The lack of effect of GDP[S], together with the potent block by GTP[S] in our system suggest that a small GTPase is involved in the vesicular transport incorporating I_{crac} channels into the plasma membrane. A similar conclusion was reached by Bird and Putney [6], although in their experiments using mouse lacrimal cells, both GTP[S] and GDP[S], injected to a final concentration of 1 – 2 mM, blocked store-dependent Ca^{2+} entry. This effect of GDP[S] compared with the lack of effect of a similar concentration in our studies or in RBL cells [7] may be due to a species difference, but is more likely to be a consequence of using intact cells and/or injecting from a concentrated stock (100 mM) in the lacrimal-cell study. In RBL cells, a factor required for activation of I_{crac} is dialysed during whole-cell recordings with a time course predicted for a small protein rather than a nucleotide or inositol phosphate [7]. In our experiments I_{crac} was also dependent upon the time from initial whole-cell break-in and ionomycin application. The time course of decay was 34.7 s $\cdot$ $M\Omega^{-1}$, i.e. approx. 650 s, taking account of the average series resistance of 19 $M\Omega$; these are similar values to those reported for RBL cells. As concluded for RBL cells, the dialysed factor may therefore be a small G-protein or a protein such as a 'GAP' (GTP-ase activating protein), a 'GDI' (GDP dissociation inhibitor) or a GNRP ('guanine nucleotide release factor') involved in small G-protein function [10].

From the data presented here, we propose that, when the Ca^{2+} stores are full, the channels underlying I_{crac} are stored in an endomembrane compartment. Following store depletion, I_{crac} channels are transported from this compartment within membrane vesicles to the plasma membrane. I_{crac} appears once the vesicles have fully fused with the plasma membrane. Endocytic retrieval of I_{crac} channels from the plasma membrane, back to its store, may also account for reduction of I_{crac} once the Ca^{2+} stores refill, although we have no evidence for the mechanism controlling closure of I_{crac} channels. On the basis of the actions of primaquine in a reconstituted secretion system, this drug most likely blocks appearance of I_{crac} by preventing the formation of I_{crac} -containing vesicles [12].

In conclusion, we have shown that two established inhibitors of vesicle-mediated protein transport, GTP[S] and primaquine, block the appearance of Ca^{2+} influx currents in response to Ca^{2+} store depletion in rat megakaryocytes. Primaquine is far less effective if added after I_{crac} has developed. Thus the channels underlying I_{crac} may be held in a membrane compartment and transported to the plasma membrane following release of internal Ca^{2+} .

This work was funded by grants from the Biotechnology and Biological Sciences Research Council and the British Heart Foundation (BHF). M.P.M.-S. holds a BHF Science Lectureship. We thank Dr. S. O. Sage for pre-submission comments on the manuscript.

REFERENCES

- 1 Hoth, M. and Penner, R. (1992) *Nature (London)* **355**, 353–355
- 2 Somasundaram, B. and Mahaut-Smith, M. P. (1994) *J. Physiol. (London)* **480**, 225–231
- 3 Fasolato, C., Innocenti, B. and Pozzan, T. (1994) *Trends Pharmacol. Sci.* **15**, 77–83
- 4 Putney, J. W. (1986) *Cell Calcium* **7**, 1–12
- 5 Irvine, R. F. (1992) *FASEB J.* **6**, 3085–3091
- 6 Bird, G. St. J. and Putney, J. W., Jr. (1993) *J. Biol. Chem.* **268**, 21486–21488
- 7 Fasolato, C., Hoth, M. and Penner, R. (1993) *J. Biol. Chem.* **268**, 20737–20740
- 8 Randriamampita, C. and Tsien, R. Y. (1992) *Nature (London)* **364**, 809–814
- 9 Sargeant, P. and Sage, S. O. (1994) *Pharmacol. Ther.* **64**, 395–443
- 10 Pryer, N. K., Wuestehube, L. J. and Schekman, R. (1992) *Annu. Rev. Biochem.* **61**, 471–516
- 11 Bradbury, N. A. and Bridges, R. J. (1994) *Am. J. Physiol.* **267**, C1–C24
- 12 Hiebsch, R. R., Raub, T. J. and Wattenberg, B. W. (1991) *J. Biol. Chem.* **266**, 20323–20328
- 13 Schwartz, A. L., Bolognesi, A. and Fridovich, S. E. (1984) *J. Cell Biol.* **98**, 732–738
- 14 Braulke, T., Geuze, H. J., Slot, J. W., Hasilik, A. and von Figura, K. (1986) *Eur. J. Cell Biol.* **43**, 316–321
- 15 Hoth, M. and Penner, R. (1993) *J. Physiol. (London)* **465**, 359–386
- 16 Strous, G. J., DuMaine, A., Zijderhand-Bleekemolen, J. E., Slot, J. W. and Schwartz, A. J. (1985) *J. Cell Biol.* **101**, 531–539
- 17 Harrison, P. T., Davis, W., Norman, J. C., Hockaday, A. R. and Allen, J. M. (1994) *J. Biol. Chem.* **269**, 24396–24402
- 18 Sewell, J. L. and Kahn, R. A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4620–4624
- 19 Melancon, P., Glick, B. S., Malhotra, V. et al. (1987) *Cell* **51**, 1053–1062
- 20 Eckstein, F., Cassell, D., Levkowitz, H., Lowe, M. and Selinger, Z. (1979) *J. Biol. Chem.* **254**, 9829–9834