Store-activated Ca^{2+} inflow in *Xenopus laevis* oocytes: inhibition by primaquine and evaluation of the role of membrane fusion

Roland B. GREGORY* and Greg J. BARRITT

Department of Medical Biochemistry, School of Medicine, Faculty of Health Sciences, Flinders University, G.P.O. Box 2100, Adelaide, South Australia, 5001, Australia

The role of membrane fusion in the activation of store-activated Ca^{2+} channels (SACCs) in the plasma membrane of *Xenopus laevis* oocytes was investigated with primaquine, an inhibitor of vesicle trafficking, reagents that disrupt the cytoskeleton, and reagents that activate or inhibit the functions of monomeric and trimeric GTP-binding regulatory proteins. Ca^{2+} inflow was assessed by measuring the rate of increase in the fluorescence of the intracellular Ca^{2+} chelator fluo-3 after the addition of extracellular Ca^{2+} to oocytes previously incubated in the absence of added Ca^{2+} . Primaquine inhibited the 3-deoxy-3-fluoro-Ins(1,4,5) P_3 (Ins(1,4,5) P_3 F)-stimulated increase in Ca^{2+} -induced fluo-3 fluorescence with no detectable effect on the release of Ca^{2+} from intracellular stores. The effect of primaquine was observed within 1.5 min, showed similarity to the inhibition induced by Gd^{3+} , was reversible, and was observed when primaquine was added either before or after activation of the SACCs. The degree of inhibition of Ca^{2+} inflow by primaquine was halved when the extracellular concentration of Ca^{2+} was increased from 3.1 to 12.5 mM. Primaquine also inhibited Ca^{2+} inflow through cholera toxin-activated divalent cation channels

and *Drosophila* Trpl channels (expressed in oocytes after injection of *trpl* cRNA). These results indicate that primaquine inhibits open SACCs, possibly by directly inhibiting Ca^{2+} flow through the channel pore. Colchicine plus cytochalasin B, Brefeldin A, the peptide Arf-1 (2–17) (introduced by microinjection), lovastatin or pertussis toxin did not inhibit the Ins(1,4,5) P_3 F-stimulated increase in fluo-3 fluorescence. In contrast, guanosine 5'-[γ -thio]triphosphate (GTP[S]), guanosine 5'-[β , γ -imido]triphosphate (p[NH]ppG) and AlF_4^- , but not guanosine 5'-[β -thio]diphosphate, inhibited the Ins(1,4,5) P_3 F-stimulated increase in fluo-3 fluorescence. Co-administration of GTP did not prevent the inhibition by GTP[S] or AlF_4^- . Staurosporine largely prevented the inhibition of store-activated Ca^{2+} inflow by GTP[S]. It is concluded that membrane fusion processes are unlikely to be involved in the link between the release of Ca^{2+} from the endoplasmic reticulum and activation of SACCs. The idea that this link is achieved by direct interaction of a protein(s) in the endoplasmic reticulum membrane with the SACC protein is briefly discussed.

INTRODUCTION

Receptor-activated Ca^{2+} channels are essential to the actions of hormones and growth factors on non-excitabile and some excitable cells [1]. On the basis of cation specificity and the likely mechanism of activation, three groups of receptor-activated Ca^{2+} channels have been identified: ligand-gated channels, intracellular messenger-activated channels and store-activated channels [2]. The last-mentioned process, initially described by Putney [3], involves the release of Ca^{2+} from a component of the smooth endoplasmic reticulum (SER), which in turn leads to activation of Ca^{2+} inflow across the plasma membrane. This form of receptor-activated Ca^{2+} channel is prevalent in many types of animal cell and is important in increases in intracellular Ca^{2+} concentration promoted by extracellular signals [4].

Neither the structures nor the mechanisms of activation of store-activated calcium channels (SACCs) are well understood [4]. Three mechanisms have emerged to explain how the release of Ca^{2+} from a region of the SER leads to activation of the plasma membrane SACCs. These are: (1) conformational coupling between one or more proteins in the SER and the SACCs [5], (2) the formation and action of a mobile intracellular messenger [6–8], and (3) the transport of vesicles containing SACCs from an intracellular site to the plasma membrane where the SACCs are inserted into the plasma membrane by fusion of the vesicle and plasma membranes [2,9] (compare the insertion of glucose

transporters in the plasma membrane [10]). Evidence for the vesicle-mediated insertion hypothesis comes directly from experiments with primaquine and chloroquine, which inhibit store-activated Ca^{2+} inflow in megakaryocytes [9], and indirectly from the observations that the activation of SACCs requires a monomeric and/or trimeric GTP-binding regulatory protein (G-protein) [11–14]. Primaquine and chloroquine inhibit vesicle budding and fusion in a number of intracellular pathways such as protein secretion and recycling of plasma membrane receptors [15,16], but also inhibit the activities of several membrane-associated enzymes [17–19], as well as affecting membrane properties [20]. G-proteins are known to be required for membrane fusion events [21,22].

In the present experiments we have tested the vesicle-mediated insertion hypothesis for the activation of SACCs in *Xenopus laevis* oocytes by using primaquine, agents that inhibit or activate G-protein function, and reagents that disrupt the cytoskeleton because normal integrity of the cytoskeleton is required for vesicle trafficking [23]. *X. laevis* oocytes possess well-characterized store-activated Ca^{2+} channel(s) [24,25] and their large size allows the ready introduction of reagents to the cytoplasmic space. It was found that primaquine inhibits store-activated Ca^{2+} inflow in *Xenopus* oocytes. However, this inhibition is due to a direct interaction of primaquine with SACCs and not to an inhibition of membrane fusion. Moreover, reagents that disrupt the functions of G-proteins or the cytoskeleton did not inhibit the

Abbreviations used: Ca^{2+}_o , extracellular Ca^{2+} ; $[\text{Ca}^{2+}]_o$, extracellular Ca^{2+} concentration; 'caged' Ins(1,4,5) P_3 , Ins(1,4,5) P_3 $P^{4(5)}$ -1-(2-nitrophenyl)ethyl ester; GDP[S], guanosine 5'-[β -thio]diphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; Ins(1,4,5) P_3 F, 3-deoxy-3-fluoro-Ins(1,4,5) P_3 ; p[NH]ppG, guanosine 5'-[β , γ -imido]triphosphate; SACC, store-activated calcium channel; SER, smooth endoplasmic reticulum.

* To whom correspondence should be addressed.

activation of SACCs so that it is concluded that membrane fusion is not required for the activation of SACCs in *Xenopus* oocytes.

MATERIALS AND METHODS

Materials

Primaquine, chloroquine, Brefeldin A, pertussis toxin, cytochalasin B, colchicine and staurosporine were from Sigma-Aldrich (Castle Hill, N.S.W., Australia); cholera toxin was from Sapphire Bioscience (Alexandria, N.S.W., Australia); and desferrioxamine mesylate (desferoxamine) was from Ciba-Geigy Australia (Lane Cove, N.S.W., Australia). Lovastatin was a gift from Merck, Sharp and Dohme. The synthesis of *Drosophila trp* cRNA and expression in *Xenopus* oocytes were performed as described previously [26]. All other reagents and *X. laevis* were obtained from the sources described previously [14,26]. Peptide Arf-1 (2–17) was synthesized by Dr. B. Kemp (St. Vincent's Institute of Medical Research, Melbourne, Victoria, Australia).

Methods

The isolation of oocytes (stages V and VI) from wild-type and albino *X. laevis* and the incubation of oocytes were performed as described previously [26,27]. Microinjection of agents into oocytes, measurement of the fluorescence of oocytes loaded with fluo-3, determination of initial rates of increase in Ca^{2+}_o -induced fluo-3 fluorescence, and the photolysis of $\text{Ins}(1,4,5)\text{P}_3$, $\text{P}^{1(5)}$ -1-(2-nitrophenyl)ethyl ester ('caged' $\text{Ins}(1,4,5)\text{P}_3$) were performed as described previously [26,27]. A period of 20 min was allowed for recovery of the oocytes after microinjection. Estimated intracellular concentrations of fluo-3, 3-deoxy-3-fluoro- $\text{Ins}(1,4,5)\text{P}_3$ [$\text{Ins}(1,4,5)\text{P}_3\text{F}$] and 'caged' $\text{Ins}(1,4,5)\text{P}_3$ were 225–240, 12–16 and 12.5 μM respectively. The duration of each photolysis event was less than 1 s.

Analysis of results

Except where indicated otherwise, the results are the means $\pm$ S. E. M. for the number of oocytes indicated in parenthesis.

RESULTS

Effects of primaquine

The addition of extracellular Ca^{2+} (Ca^{2+}_o) to oocytes loaded with fluo-3 and $\text{Ins}(1,4,5)\text{P}_3\text{F}$, and preincubated in the absence of added Ca^{2+}_o , caused a large increase in fluorescence (Figure 1) due to $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated Ca^{2+} inflow [26,28]. After the addition of Ca^{2+}_o , the initial rate of increase in fluo-3 fluorescence is proportional to the rate of Ca^{2+} inflow. At later times after the addition of Ca^{2+}_o , the rate of change of fluo-3 fluorescence represents the balance between the rate of Ca^{2+} inflow and the rate of Ca^{2+} removal from the cytoplasm. Primaquine (1 mM), added at the same time as Ca^{2+}_o , markedly inhibited this Ca^{2+}_o -induced increase in fluo-3 fluorescence (Figure 1, upper panel). The effect of primaquine was clearly evident within approx. 1.5 min (Figure 1, upper panel). The initial rate of Ca^{2+}_o -induced increase in fluo-3 fluorescence in the absence of primaquine, 4.34 ± 0.52 fluorescence units/min, was decreased by almost 75 % to 1.12 ± 0.11 fluorescence units/min in the presence of primaquine.

The effects of primaquine on the $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated Ca^{2+}_o -induced increase in fluorescence were compared with those of Gd^{3+} , a potent inhibitor of SACCs [27,29]. As expected, Gd^{3+}

Figure 1 Effect of primaquine and Gd^{3+} on the $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated Ca^{2+}_o -induced increase in fluo-3 fluorescence

Oocytes from albino (upper panel) or wild-type (lower panel) *Xenopus* were microinjected with $\text{Ins}(1,4,5)\text{P}_3\text{F}$ and fluo-3 and then placed in Ca^{2+} -free medium, after which fluorescence was measured with a spectrofluorimeter, as described in the Materials and methods section. The arrows indicate the times of addition of CaCl_2 (final concentration 6.25 mM) (●) in both the upper and lower panels, or the time of addition of CaCl_2 and primaquine (1 mM) (■) in the upper panel or the time of addition of CaCl_2 and GdCl_3 (60 μM) (▲) in the lower panel. The values are the means $\pm$ S.E.M. of results obtained with five to twelve oocytes and are representative of two similar experiments.

(60 μM) inhibited the $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated Ca^{2+}_o -induced increase in fluorescence and the effect was evident within 1.2 min, this time being the period between Gd^{3+} addition and the first subsequent fluorescence reading (Figure 1, lower panel). The initial rate of increase in Ca^{2+}_o -induced fluo-3 fluorescence in the absence of Gd^{3+} , 2.79 ± 0.26 ($n = 12$) fluorescence units/min, was decreased by 72 % to 0.78 ± 0.13 ($n = 10$) fluorescence units/min in the presence of Gd^{3+} .

Primaquine also inhibited the basal rate of Ca^{2+}_o -induced increase in fluo-3 fluorescence [absence of $\text{Ins}(1,4,5)\text{P}_3\text{F}$] (Table 1). Preincubation of oocytes with primaquine (1 mM) for 5.5 h before the injection of fluo-3 and $\text{Ins}(1,4,5)\text{P}_3\text{F}$ (results not shown) inhibited the $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+}_o -induced increase in fluo-3 fluorescence by approx. 90 %. Half-maximal inhibition of the $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated Ca^{2+}_o -induced increase in fluo-3 fluorescence was observed at a primaquine concentration of 0.3 mM. Chloroquine (1 mM) inhibited the $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -

Table 1 Inhibition by primaquine of Ca^{2+} inflow through the cholera toxin-activated divalent cation channels and the *Drosophila* *Trpl* divalent cation channels

Measurement of initial rates of increase in fluo-3 fluorescence {induced by addition of 6.25 mM Ca^{2+}_o [for *trpl* cRNA-injected oocytes] or 12.5 mM Ca^{2+}_o [for cholera toxin-treated (8 $\mu\text{g}/\text{ml}$ for 24 h) oocytes]} and microinjection of *Drosophila trpl* cRNA were performed as described in the Materials and methods section. Primaquine was added before the microinjection of fluo-3. The results are the means $\pm$ S.E.M. for the number of oocytes shown in parenthesis. Degrees of significance, determined with Student's *t* test, for unpaired samples were: $P < 0.001$ for cholera toxin compared with control; $P < 0.3$ for cholera toxin plus primaquine compared with control plus primaquine; $P < 0.01$ for *trpl* cRNA-injected compared with 'mock'-injected; $P < 0.05$ for *trpl* cRNA-injected plus primaquine compared with 'mock'-injected plus primaquine.

Oocyte	Agonist	Primaquine concentration (mM)	Initial rate of increase in fluo-3 fluorescence (fluorescence units/min)
Control	None	0	0.12 ± 0.03 (6)
	None	1.0	0.03 ± 0.04 (6)
	Cholera toxin	0	0.37 ± 0.05 (3)
	Cholera toxin	1.0	0.08 ± 0.04 (5)
'Mock'-injected	Ins(1,4,5) P_3 F	0	1.27 ± 0.06 (10)
	Ins(1,4,5) P_3 F	1.0	0.35 ± 0.07 (9)
<i>trpl</i> cRNA-injected	Ins(1,4,5) P_3 F	0	1.83 ± 0.27 (9)
	Ins(1,4,5) P_3 F	1.0	0.22 ± 0.04 (9)

stimulated Ca^{2+}_o -induced increase in fluo-3 fluorescence by 30 % ($n = 8$) (results not shown).

To assess whether inhibition of the Ins(1,4,5) P_3 F-stimulated increase in Ca^{2+}_o -induced fluo-3 fluorescence by primaquine was reversible, oocytes were incubated for 2.5 h in the presence of 1 mM primaquine before the microinjection of Ins(1,4,5) P_3 F and fluo-3. Half of the oocytes were then maintained in primaquine-free media and the other half were incubated in the presence of 1 mM primaquine. The rates of increase in Ca^{2+}_o -induced fluorescence were 0.61 ± 0.08 ($n = 10$) for oocytes exposed to primaquine for the full incubation period, 2.14 ± 0.28 ($n = 9$) for oocytes incubated with primaquine in the initial phase, and 3.65 ± 0.23 ($n = 12$) (all in fluorescence units/min) for oocytes not exposed to primaquine at any time. Thus a considerable proportion of the effect of primaquine could be reversed.

The degree of inhibition by primaquine of the Ins(1,4,5) P_3 F-stimulated increase in Ca^{2+}_o -induced fluo-3 fluorescence decreased when the $[\text{Ca}^{2+}]_o$ was increased from 3.1 to 12.5 mM. At 3.1 mM Ca^{2+}_o , the rates of the Ins(1,4,5) P_3 F-stimulated increase in Ca^{2+}_o -induced fluo-3 fluorescence were 0.57 ± 0.07 ($n = 10$) and 3.13 ± 0.33 ($n = 10$) (fluorescence units/min) in the presence and absence of primaquine respectively. At 12.5 mM Ca^{2+}_o , the corresponding rates were 3.26 ± 0.21 ($n = 11$) and 5.57 ± 0.44 ($n = 10$) respectively. This represents degrees of inhibition by primaquine of the Ins(1,4,5) P_3 F-stimulated increase in Ca^{2+}_o -induced fluo-3 fluorescence of 82 % and 42 % at 3.1 and 12.5 mM $[\text{Ca}^{2+}]_o$ respectively.

Primaquine had no detectable effect on the release of Ca^{2+} from the SER as assessed by measuring the transient increase in fluorescence that followed the photolysis of 'caged' Ins(1,4,5) P_3 in oocytes loaded with fluo-3 and incubated in the absence of added Ca^{2+}_o (Figure 2, upper panel, curves 1 and 2; compare Figure 2, lower panel, curves 1 and 2). The fluorescence increase induced by the photolysis of 'caged' Ins(1,4,5) P_3 in a number of oocytes was quantified by measuring the area beneath the fluorescence trace generated by a photolysis event and calculating the ratio of the area of the fluorescence peak (mV $\cdot$ min) to the

Figure 2 Effect of primaquine on the release of Ca^{2+} from intracellular stores and Ca^{2+} inflow induced by the photolysis of 'caged' Ins(1,4,5) P_3

Albino oocytes were microinjected with 'caged' Ins(1,4,5) P_3 , and fluo-3 and fluorescence measurements performed on individual oocytes with a fluorescence microscope, as described in the Materials and methods section. Breaks in the traces correspond to short intervals when light incident on the photomultiplier was blocked in order to photolyse 'caged' Ins(1,4,5) P_3 (indicated by the arrows). Upper panel: oocytes were initially present in Ca^{2+} -free medium. CaCl_2 (6.6 mM) was added at 10.5 min and primaquine (1 mM) at 22 min, as indicated by the horizontal bars. Lower panel: oocytes, initially present in Ca^{2+} -free medium, were exposed to primaquine (1 mM) 6 min before the first photolysis event. Calcium (6.6 mM) was added as indicated by the horizontal bar. In both panels the traces shown were obtained with a single oocyte and are representative of the results obtained for three oocytes.

height of the fluorescence peak (mV). The values for this ratio were 1.31 ± 0.10 ($n = 16$), 1.30 ± 0.08 ($n = 7$) and 1.14 ± 0.14 ($n = 3$) for oocytes incubated in the absence of primaquine and in the presence of 0.5 and 1.0 mM primaquine respectively. Photolysis of 'caged' Ins(1,4,5) P_3 in control oocytes incubated in the presence of Ca^{2+}_o (6.6 mM) caused a more prolonged increase in fluorescence (Figure 2, upper panel, curve 3; compare curves 1 and 2) owing to the stimulation by Ins(1,4,5) P_3 of Ca^{2+} inflow as well as Ca^{2+} release from the SER. The ratio of peak area to peak height was 5.28 ± 0.65 ($n = 12$) which is 4-fold higher than the value obtained in the absence of added Ca^{2+}_o . The prolonged increase in fluorescence observed in the presence of Ca^{2+}_o was markedly reduced by primaquine (Figure 2, upper panel, curve 4; compare curve 3; also Figure 2, lower panel, curves 3, 4 and 5; compare Figure 2, upper panel, curve 3). The ratio of peak

Figure 3 Comparison of the effects of primaquine and Gd^{3+} on the fluorescence of oocytes loaded with fluo-3 and $Ins(1,4,5)P_3F$ and incubated in the presence of Ca^{2+} .

Oocytes were microinjected with $Ins(1,4,5)P_3F$ and fluo-3 and then placed in Ca^{2+} -free medium, after which fluorescence was measured with a spectrofluorimeter, as described in the Materials and methods section. $CaCl_2$ (6.25 mM) and medium (●), primaquine (■) or $GdCl_3$ (▲) were added as indicated by the arrows. The final concentrations of primaquine and $GdCl_3$ were 1 mM and 60 μM respectively. The results shown are the means $\pm$ S.E.M. of those obtained for 11 ($CaCl_2$ alone) or 12 (primaquine or Gd^{3+} present) oocytes and are representative of those obtained in three similar experiments.

area to peak height was 2.74 ± 0.38 ($n = 9$) in the presence of 6.6 mM Ca^{2+} and 1 mM primaquine. By Student's t test for unpaired samples, $P < 0.001$ for the ratio in the presence of 6.6 mM Ca^{2+} and 1 mM primaquine compared with the ratio in the presence of 6.6 mM Ca^{2+} .

The results above indicate that primaquine inhibits Ca^{2+} inflow when added either before activation of the SACCs [2.5 or 5.5 h before injection of $Ins(1,4,5)P_3F$] or when the SACCs are already activated [addition of primaquine 30 min after $Ins(1,4,5)P_3F$ injection]. Thus the effect of primaquine might be either on the process that activates the SACCs or on the movement of Ca^{2+} through the SACCs. To investigate the second possibility further, the effects of primaquine added when the Ca^{2+} -induced increase in fluorescence had reached a plateau were compared with those of Gd^{3+} . The addition of either primaquine or Gd^{3+} caused a decrease in fluorescence that, after approx. 20 min, approached a steady-state value approx. 10% greater than the initial (zero time) value (Figure 3). The time course for the effect of primaquine was identical with that for Gd^{3+} (Figure 3).

The effects of primaquine on two other divalent cation channels, the cholera toxin-activated divalent cation channel previously identified in *Xenopus* oocytes [30] and the *Drosophila* Trpl-, Ca^{2+} - and $Ins(1,4,5)P_3$ -activated non-selective cation channel [31] expressed in *Xenopus* oocytes [26], were investigated. Primaquine inhibited cholera toxin-stimulated Ca^{2+} inflow (Table 1). In oocytes expressing the Trpl protein, the rate of the $Ins(1,4,5)P_3F$ -stimulated increase in Ca^{2+} -induced fluo-3 fluorescence was 44% higher than that in 'mock'-injected oocytes (Table 1). Primaquine inhibited the rate of the $Ins(1,4,5)P_3F$ -stimulated increase in Ca^{2+} -induced fluo-3 fluorescence in both 'mock'- and Trpl cRNA-injected oocytes (Table 1).

Effects of inhibitors and activators of G-protein function

GTP[S] (50 μM), introduced by microinjection, inhibited the $Ins(1,4,5)P_3F$ -stimulated increase in fluo-3 fluorescence by

Figure 4 Inhibition of the $Ins(1,4,5)P_3F$ -stimulated Ca^{2+} -induced increase in fluo-3 fluorescence by GTP[S] and prevention of GTP[S] inhibition by staurosporine

Albino oocytes were microinjected with either $Ins(1,4,5)P_3F$ or $Ins(1,4,5)P_3F$ and GTP[S] after 4 h of preincubation with either 0.2% (v/v) DMSO or 200 nM staurosporine; they were then placed in Ca^{2+} -free medium and fluorescence was measured with a spectrofluorimeter, as described in the Materials and methods section. Staurosporine or DMSO was also present after microinjection. $CaCl_2$ (6.25 mM) was added at the time indicated by the arrow. ○, no GTP[S], staurosporine present; ●, no GTP[S], DMSO present; ▲, GTP[S] and staurosporine; ■, GTP[S] and DMSO. The values are the means $\pm$ S.E.M. for four to six oocytes and are representative of those obtained in four experiments. The estimated intracellular concentration of GTP[S] was 50 μM .

50–60% (Figure 4 and Table 2), as shown previously [32]. At 150 μM GTP[S] the degree of inhibition was 85% (results not shown). p[NH]ppG, NaF, and $AlCl_3$ plus NaF (AlF_4^-), but not guanosine 5'-[β -thio]diphosphate (GDP[S]) or GTP, also inhibited the $Ins(1,4,5)P_3F$ -stimulated increase in fluo-3 fluorescence (Table 2). Deferoxamine, which chelates trivalent cations such as Al^{3+} [33], largely prevented the effect of $AlCl_3$ plus NaF (Table 2). The inhibition by GTP[S] and AlF_4^- was not prevented by the co-injection of GTP (Table 2). However, as shown by Petersen and Berridge [32], the effect of GTP[S] was largely prevented by staurosporine, an inhibitor of protein kinase C [34] (Table 2 and Figure 4).

No detectable inhibition of $Ins(1,4,5)P_3F$ -stimulated Ca^{2+} inflow was observed when oocytes were preincubated for: 27 h with pertussis toxin, which ADP-ribosylates G_i , G_o and G_t (transducin) [35]; 24 h with lovastatin, which inhibits the isoprenylation of some monomeric G-proteins [36,37]; or 5 h with Brefeldin A, an inhibitor of membrane fusion events involving Arf proteins [38] (results not shown). The peptide Arf-1 (2–17), which inhibits the functions of Arf proteins, including the budding of transport vesicles involved in protein trafficking [39], also had no effect on store-activated Ca^{2+} inflow (results not shown).

Effects of cytochalasin B and colchicine

Preincubation of oocytes with colchicine plus cytochalasin B for 18 h did not substantially inhibit $Ins(1,4,5)P_3F$ -stimulated Ca^{2+} inflow. The rates of $Ins(1,4,5)P_3F$ -stimulated increase in Ca^{2+} -induced fluo-3 fluorescence in the presence and absence of cytochalasin B and colchicine (16 μM each) were 1.85 ± 0.18 ($n = 13$) and 2.15 ± 0.16 ($n = 10$) fluorescence units/min respectively. Cytochalasin B plus colchicine did block the

Table 2 Inhibition of $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated Ca^{2+} inflow by $\text{GTP}[\text{S}]$, $\text{p}[\text{NH}]\text{ppG}$ and AlF_4^-

The nucleotides, NaF, AlCl_3 and NaCl (intracellular concentrations shown) were co-injected into albino *Xenopus* oocytes with fluo-3 and $\text{Ins}(1,4,5)\text{P}_3\text{F}$. Staurosporine and deferoxamine (extracellular concentrations shown) were added to the incubation medium 4 and 2 h respectively before microinjection of $\text{Ins}(1,4,5)\text{P}_3\text{F}$ and fluo-3, and were also present during the measurement of fluorescence analysis. Initial rates of increase in fluo-3 fluorescence were measured after the addition of 6.25 mM Ca^{2+}_o to oocytes previously incubated in the absence of added Ca^{2+}_o . In the absence of $\text{Ins}(1,4,5)\text{P}_3\text{F}$, the Ca^{2+}_o -induced increase in fluorescence was 0.18 ± 0.02 ($n = 10$) fluorescence units/min. The values shown are the means $\pm$ S.E.M. for the number of individual oocytes shown in parentheses and are derived from three series of experiments. Degrees of significance, assessed with Student's t test for unpaired samples, were: $P < 0.001$ for comparison of $\text{GTP}[\text{S}]$, $\text{p}[\text{NH}]\text{ppG}$ and $\text{GTP}[\text{S}]/\text{GTP}$ with control (no nucleotide injection); $P < 0.001$ for $\text{GTP}[\text{S}]$ compared with control and for staurosporine/ $\text{GTP}[\text{S}]$ compared with staurosporine and for staurosporine/ $\text{GTP}[\text{S}]$ with $\text{GTP}[\text{S}]$; $P < 0.001$ for NaF compared with NaCl or NaF plus AlCl_3 compared with NaCl plus AlCl_3 ; $P < 0.01$ for NaF plus AlCl_3 with deferoxamine compared with NaF plus AlCl_3 without deferoxamine.

Agent under test	Initial rate of $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated increase in fluo-3 Fluorescence (fluorescence units/min)
None	4.30 ± 0.15 (63)
$\text{GTP}[\text{S}]$ (50 μM)	2.21 ± 0.13 (29)
$\text{p}[\text{NH}]\text{ppG}$ (200 μM)	2.05 ± 0.23 (13)
$\text{GDP}[\text{S}]$ (50 μM)	3.99 ± 0.36 (20)
$\text{GTP}[\text{S}]$ (50 μM) plus GTP (500 μM)	1.56 ± 0.18 (7)
GTP (500 μM)	3.89 ± 0.35 (8)
None	5.82 ± 0.38 (16)
$\text{GTP}[\text{S}]$ (50 μM)	2.64 ± 0.15 (15)
Staurosporine (200 nM)	4.83 ± 0.33 (19)
Staurosporine (200 nM) plus $\text{GTP}[\text{S}]$ (50 μM)	3.64 ± 0.26 (19)
NaCl (5 mM)	4.69 ± 0.23 (26)
NaF (5 mM)	3.02 ± 0.20 (25)
NaCl (5 mM) plus AlCl_3 (15 μM)	4.34 ± 0.23 (13)
NaF (5 mM) plus AlCl_3 (15 μM)	2.59 ± 0.26 (15)
NaF (5 mM) plus AlCl_3 (15 μM) plus GTP (1 mM)	2.23 ± 0.38 (4)
NaCl (5 mM) plus AlCl_3 (15 μM) plus deferoxamine (100 μM)	3.99 ± 0.26 (11)
NaF (5 mM) plus AlCl_3 (15 μM) plus deferoxamine (100 μM)	3.59 ± 0.37 (9)

stimulation of Ca^{2+} inflow [27] and the loss of black pigmentation from the animal hemisphere induced by a high concentration of $\text{GTP}[\text{S}]$ (0.8 mM) (results not shown), indicating that these agents had disrupted the cytoskeleton.

DISCUSSION

Action of primaquine

The results show that primaquine rapidly and reversibly inhibits $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated Ca^{2+} inflow in *Xenopus* oocytes without a detectable effect on the release of Ca^{2+} from intracellular stores. This last result indicates that the effects of primaquine are unlikely to be due to the quenching of fluo-3 fluorescence or the chelation of intracellular Ca^{2+} . Two explanations for the primaquine-induced inhibition of $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated Ca^{2+} inflow (assessed by measurement of the initial rate of Ca^{2+}_o -induced increase in fluo-3 fluorescence) are that primaquine either directly inhibits the flow of Ca^{2+} through open SACCs or that it inhibits the process by which Ca^{2+} release from the SER activates SACCs. In relation to the second explanation, it has been proposed that the activation of SACCs by Ca^{2+} release from the SER involves the movement of vesicles that contain SACCs to the plasma membrane and insertion of SACCs into the plasma membrane by fusion of the vesicle and the plasma membranes

[2,9]. Inactivation of SACCs might then involve endocytotic retrieval from the plasma membrane [9]. If this were the mechanism of activation of SACCs, the addition of primaquine before $\text{Ins}(1,4,5)\text{P}_3\text{F}$ might inhibit SACC activation by inhibiting the formation of transport vesicles [40] and hence the insertion of SACCs into the plasma membrane. The addition of primaquine after $\text{Ins}(1,4,5)\text{P}_3\text{F}$ might reverse SACC activation if the endocytosis of SACCs already in the plasma membrane continued in the presence of primaquine.

Several observations indicate that primaquine inhibits Ca^{2+} movement through the SACC pore rather than a step in SACC activation. (1) The action of primaquine was not specific for SACCs. Thus primaquine inhibited basal (no agonist) Ca^{2+} inflow and Ca^{2+} inflow through cholera toxin-activated divalent cation channels and *Drosophila* Trp1 divalent cation channels. These are not SACCs and are not activated by Ca^{2+} release from the SER [30,31]. (2) The time courses for the decrease in fluorescence caused by the addition of primaquine or Gd^{3+} , a potent inhibitor of Ca^{2+} movement through SACCs [27,29], to $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -loaded oocytes after the Ca^{2+}_o -induced increase in fluorescence had reached a plateau were almost identical. In this experiment the decrease in fluorescence after the inhibition of Ca^{2+} inflow is due to continued removal of Ca^{2+} from the cytoplasmic space by the plasma membrane and SER (Ca^{2+} + Mg^{2+})ATPases [41]. (3) The rate of decrease in fluorescence at room temperature after primaquine addition at the fluorescence plateau [as described in (2)] was faster than predicted if endocytosis (of the Ca^{2+} channel protein) were to determine the net rate of inhibition by primaquine of Ca^{2+} inflow [15,42]. (4) Either primaquine or Gd^{3+} , added together with Ca^{2+}_o , rapidly (within 1.5 min) caused similar degrees of inhibition of the $\text{Ins}(1,4,5)\text{P}_3\text{F}$ -stimulated increase in fluo-3 fluorescence. (5) The degree of inhibition induced by primaquine was decreased at a higher $[\text{Ca}^{2+}]_o$, indicating that primaquine and Ca^{2+}_o bind to the same target protein. Although the possibility that primaquine inhibits one or more steps in the pathway of activation of SACCs as well as inhibiting Ca^{2+} flow through the SACC pore cannot be completely eliminated, it is considered unlikely. Moreover the conclusion that primaquine interacts with the SACC protein is consistent with reports that primaquine has a wide range of effects on intracellular processes [16–18,20,42,43].

The conclusions just described differ from that reached by Somasundaram et al. [9] in a study of primaquine inhibition of I_{CRAC} (calcium-release-activated current) in rat megakaryocytes. Those authors concluded that the most likely explanation for the effect of primaquine was inhibition of trafficking and recycling of SACCs, although they could not eliminate a direct inhibition by primaquine of the SACCs. The differences in interpretation of the results obtained for megakaryocytes and oocytes might reflect differences in the mechanisms of activation of the SACCs in the two cell types and the fact that primaquine affects several processes in intact cells [16–18,20,42,43].

Role of membrane fusion in the activation of SACCs

As part of a test of the vesicle-mediated membrane insertion hypothesis for the activation of SACCs, reagents that interfere with the functions of G-proteins were employed to determine whether a trimeric and/or a monomeric G-protein is required in the link between Ca^{2+} release from the SER to the activation of SACCs because G-proteins are required for membrane fusion events [21,22]. As shown by others [11–13] it was found that $\text{GTP}[\text{S}]$ and $\text{p}[\text{NH}]\text{ppG}$ (activators of trimeric and many monomeric G-proteins, and inhibitors of the Arf monomeric G-proteins [44,45]), and AlF_4^- (an activator of trimeric but not

monomeric G-proteins [46]), inhibited store-activated Ca^{2+} inflow in oocytes. These results indicate that one or more G-proteins are required for, or regulate, the activation of SACCs. Because extensive treatment with pertussis toxin did not inhibit $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} inflow it is unlikely that the trimeric G-protein involved is pertussis toxin-sensitive (i.e. it is unlikely to be G_i , G_t or G_o).

GTP did not prevent the GTP[S] inhibition of the $\text{Ins}(1,4,5)\text{P}_3$ -stimulated increase in fluo-3 fluorescence. Reversal of GTP[S] inhibition would be expected if GTP[S] affected a monomeric G-protein [11]. Furthermore lovastatin, Brefeldin A or peptide Arf-1 (2–17) did not inhibit the $\text{Ins}(1,4,5)\text{P}_3$ -stimulated increase in fluo-3 fluorescence, and there are no reports that AlF_4^- activates or inhibits monomeric G-proteins [46]. These results indicate that a monomeric G-protein is unlikely to be the target for GTP[S], p[NH]ppG and AlF_4^- . Because staurosporine, an inhibitor of protein kinase C [34], substantially prevented GTP[S]-induced inhibition, it is concluded that the most likely interpretation of the results obtained with GTP[S], p[NH]ppG and AlF_4^- is that they each activate $\text{G}_{q/11}$, which couples to phospholipase $\text{C}\beta$, increase the formation of diacylglycerol, and activate protein kinase C, which in turn inhibits a step in the link between the release of Ca^{2+} from SER and the activation of SACCs [32]. Thus it is concluded that neither a trimeric nor a monomeric G-protein is part of the SACC activation pathway in *Xenopus* oocytes.

This last-mentioned finding provides indirect evidence that SACC activation does not involve vesicle-mediated insertion of SACC proteins into the plasma membrane. More direct evidence for this conclusion comes from the experiments conducted with primaquine and colchicine/cytochalasin B. Thus there is no evidence that the primaquine inhibition of SACC activation is due to inhibition of the budding and fusion of vesicles. Moreover the disruption of microfilaments and microtubules by colchicine/cytochalasin B, which might also be expected to disrupt membrane fusion processes [47], did not inhibit SACC activation. Thus it is concluded that it is unlikely that SACC activation in *Xenopus* oocytes involves vesicle-mediated insertion of SACC proteins into the plasma membrane. Two other possible mechanisms for SACC activation are the formation of a mobile intracellular messenger [6–8] and conformational coupling between one or more proteins in the SER and one or more proteins in the SACC [5]. *Xenopus* oocytes contain putative anatomical junctions between regions of the SER and the plasma membrane [48], as required in the conformational coupling model. However, although a mobile intracellular messenger has not been identified [49], it is currently not possible to exclude either hypothesis. Thus further investigation is required to elucidate the mechanism of activation of SACCs in *Xenopus* oocytes.

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