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Differential effect of PKA on the Ca^{2+} release kinetics of the type I and III $InsP_3$ receptors

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Abstract

The effects of protein kinase A (PKA) on the inositol 1,4,5-trisphosphate ($InsP_3$) receptor isoforms type I and type III were studied. The effects of PKA on the extent and rate constants for $InsP_3$-induced Ca^{2+} release (IICR) were different for the two isoforms. The effects of PKA on the type I isoform showed a biphasic relationship dependent upon the concentration of PKA used. At low concentrations of PKA (<50 U/ml), both the extent and rate constants for IICR increased, while at higher concentrations (>200 U/ml) the extent and rate constants decreased. The type III isoform showed only an increase in the extent of IICR and not in the rate constants. The effects of PKA on the type I $InsP_3$ receptor using single channel electrophysiological studies were also investigated. The stimulatory effect of PKA is due to an increase in conductance levels and not to a change in the mean open time of the channel.

Keywords: $InsP_3$ receptors; Ca^{2+} channel; Protein kinase A; Phosphorylation; Isoforms

The inositol 1,4,5-trisphosphate ($InsP_3$) receptor is an ER Ca^{2+} channel which opens in response to agonist-stimulated production of the second messenger $InsP_3$ [1]. There are three isoforms of this Ca^{2+} channel which show ≈70% sequence similarity [2]. The largest variation occurs within the modulatory domain which has several putative kinase-dependent phosphorylation sites [2], some of which when phosphorylated alter channel function [2].

Some studies using cerebellar membranes [4] and platelet membranes [5] have shown that PKA-dependent phosphorylation reduces the potency of $InsP_3$ to release Ca^{2+}. However, other studies in intact platelets [6], hepatocytes [7], cerebellar microsomes [8], and the purified type I $InsP_3$ receptor [3] have shown that PKA-dependent phosphorylation increases $InsP_3$-induced Ca^{2+} release (IICR). These variations have not as yet been adequately explained.

The type I isoform of the $InsP_3R$ has been shown to be phosphorylated at two specific sites (serines 1755 and 1589 for the rat receptor) [9]. The type II and III isoforms of the $InsP_3$ receptor do not contain the same two PKA-dependent phosphorylation sites seen on the type I isoform, but, other putative phosphorylation sequences exist [2]. Indeed, a study by Wojcikiewicz and Luo [10] showed that all three isoforms of the $InsP_3$ receptor could be phosphorylated by PKA but to vastly different extents. (The order of extent of PKA phosphorylation being; type I > type III ≫ type II.)

We have shown that, when Ca^{2+} efflux rates were monitored, all three isoforms of the $InsP_3$ receptor show similar IICR kinetics [11]. Here we investigate how the transient kinetics and single channel properties of IICR are affected by PKA and whether there are differences between the type I and III isoforms.

Materials and methods

Cerebellar microsomes, which contain >90% type I isoform, were prepared as described in [12]. SH-SY5Y cells (which contain >90% $InsP_3R$ type I [14]) were cultured in EMEM (Sigma) and RINm5F cells (which contain ≈80–90% $InsP_3R$ type III [13,14]) were cultured in

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RPMI1640 (Sigma). The appropriate culture media were supplemented with 10% FBS (Gibco-BRL, Paisley, UK), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg streptomycin. Confluent cells were detached from culture flasks with 155 mM NaCl, 10 mM Hepes, and 1 mM EDTA, pH 7.4, pelleted by centrifugation at 400*g* for 5 min, and washed and re-suspended in ice-cold cytosol buffer (ICB) (120 mM KCl, 2 mM KH_2PO_4, and 20 mM Hepes, pH 7.2).

Measurements of IICR were monitored by following changes in the fluorescence of Fluo-3 as described in [11,12]. For experiments using cells, they were incubated in ICB supplemented with 1.25 mM Fluo-3, 10 μM EGTA, 2 mM $MgCl_2$, and 2 mM ATP at 37 °C for 5 min. Uptake of Ca^{2+} into intracellular compartments was initiated by permeabilisation with 100 μg/ml saponin. On attainment of maximal Ca^{2+} uptake, $InsP_3$ was added. The extent of IICR was calculated as the percentage of Ca^{2+} released by $InsP_3$ compared to that released by 12.5 μg/ml A23187. Fluo-3 fluorescence changes were converted to free Ca^{2+} concentrations as described in [11]. In measuring IICR using cerebellar microsomes, the conditions were identical to those described in [12].

The transient kinetics of IICR for the permeabilised cells and cerebellar microsomes were undertaken using a stopped-flow spectrofluorimeter to monitor fluo-3 fluorescence as described in [11,12]. After Ca^{2+} accumulation, permeabilised cells or microsomes were introduced into a large syringe of a stopped-flow spectrofluorimeter and $InsP_3$ was added to the smaller syringe (drive ratio 10:1). The contents of the two syringes were rapidly mixed together and changes in fluorescence (monitored by excitation at 506 nm and detecting emission above 515 nm using a cut-off filter) were recorded. The data were then fitted to either a mono-exponential or bi-exponential equation as described in [11]. In addition, the effect of Ca^{2+} uptake was unlikely to influence the rates measured for IICR, as the rate constants for Ca^{2+} uptake with the permeabilised cells and membranes were determined to be $>0.01\,s^{-1}$ (about 50 times lower than the lowest IICR rate constant measured in this study) [11].

Single channel recordings were undertaken using the purified type I $InsP_3$ receptor from cerebellum as previously described in [15] and which was devoid of calcineurin. Bilayers (75–100 μm diameter) were formed over a hole in a Teflon partition bisecting a Teflon chamber by the method of Montal and Mueller (1972) [16] using Soybean lipids.Incorporation of the $InsP_3$ receptor was as previously described [17]. The chamber electrolyte solutions contained 500 mM KCl, 1 mM $CaCl_2$, and 40 mM HEPES, pH 7.3, on both *cis* and *trans* sides of the bilayer. Electrical connection of the membrane to the measuring equipment was achieved using reversible electrodes (Ag/AgCl). The free Ca^{2+} concentrations on both *trans* and *cis* sides of the membrane were decreased to 270 nM using HEDTA or BAPTA and $MgCl_2$ was added to bring the free Mg^{2+} concentration to 170 μM. The purified $InsP_3$ receptor (1 μg/ml) was then added to the *cis* chamber. Once the channel had incorporated into the bilayer, 10 μM $InsP_3$ and 100 μM ATP were added to the *cis* chamber and activity was observed at applied potential differences of between +20 and −20 mV using 500 mM K^+ as the current carrier. The changes in current were saved to a PC running PAT V7.0 software for single channel analysis, supplied by J. Dempster (University of Strathclyde, UK).

Results

To investigate the effects of PKA phosphorylation on the isoforms of the $InsP_3$ receptor, cerebellar microsomes and SH-SY5Y cells which predominantly express the type I isoform of the $InsP_3$ receptor and permeabilised RINm5F cells, which predominantly express the type III isoform of the $InsP_3$ receptor, were used. In all experiments involving microsomes or permeabilised cells, pre-incubation with the catalytic subunit of PKA in the presence of 2 mM ATP between 30 and 45 min was employed, prior to $InsP_3$ addition. This time period was sufficient to allow the PKA to cause its maximal effect.

Although all three isoforms responded maximally to 20 μM $InsP_3$ [11], the concentration required to induce a half-maximal response was substantially different (i.e., EC_{50} for type I and type III isoforms are ≈0.3 μM $InsP_3$ and ≈2 μM $InsP_3$, respectively [11,12]).

Fig. 1A shows the effects of the catalytic subunit of PKA on the extent of Ca^{2+} release from cerebellar microsomes (expressed as a percentage of that mobilized by A23187) induced by either maximal (20 μM) or half-maximal (0.3 μM) concentrations of $InsP_3$. At submaximal concentrations of $InsP_3$, the PKA has a dose-dependent biphasic effect on the extent of IICR. There was a 35% increase in the extent of IICR at

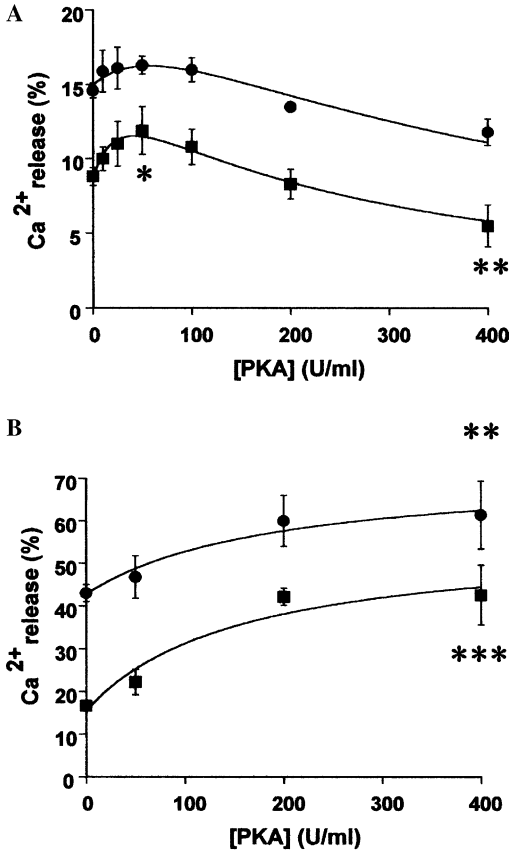

Fig. 1. Effects of PKA on the extent of IICR from the type I and type III isoforms of the $InsP_3$ receptor. (A) The effects of PKA (0–400 U/ml) on the extent of IICR from cerebellar microsomes (type I isoform) using 0.3 μM (■) and 20 μM (●) $InsP_3$, expressed as a percentage of Ca^{2+} released by A23187. (B) The effects of PKA (0–400 U/ml) on the extent of IICR from permeabilised RINm5F cells (type III isoform) using 2 μM (■) and 20 μM (●) $InsP_3$, expressed as a percentage of Ca^{2+} released by A23187. Data points marked with a *, **, and ***, have a probability of being statistically significant from the control of $P < 0.05$, $P < 0.02$, and $P < 0.01$, respectively.

Table 1
Parameters for the transient kinetics of IICR in the presence of PKA

Isoform	PKA (U/ml)	k_{obs} fast (s^{-1})	k_{obs} slow (s^{-1})	Rel. amplitude (fast)	Rel. amplitude (slow)
At 20 μM $InsP_3$					
Type I	0	2.0 ± 0.3	0.35 ± 0.03	0.57 ± 0.05	0.43 ± 0.05
(cerebellar)	50	4.1 ± 0.4	0.52 ± 0.05	0.62 ± 0.06	0.52 ± 0.08
	200	2.3 ± 0.2	0.32 ± 0.04	0.56 ± 0.03	0.34 ± 0.05
Type I	0	1.05 ± 0.05	—	1.00 ± 0.05	—
(SH-SY5Y)	50	1.85 ± 0.05	—	1.20 ± 0.05	—
	200	N.D.	—	1.02 ± 0.07	—
Type III	0	0.95 ± 0.05	—	1.00 ± 0.05	—
(RINm5F)	50	0.95 ± 0.05	—	1.15 ± 0.05	—
	200	0.85 ± 0.10	—	1.45 ± 0.10	—
At 0.3 μM $InsP_3$ (for type I) and 2 μM $InsP_3$ (for type III)					
Type I	0	0.8 ± 0.1	0.17 ± 0.05	0.35 ± 0.04	0.19 ± 0.04
(cerebellar)	50	1.5 ± 0.1	0.24 ± 0.04	0.54 ± 0.05	0.28 ± 0.03
	200	1.0 ± 0.2	0.15 ± 0.04	0.33 ± 0.03	0.19 ± 0.02
Type III	0	0.45 ± 0.03	—	0.40 ± 0.02	—
(RINm5F)	50	0.46 ± 0.03	—	0.51 ± 0.03	—
	200	0.45 ± 0.05	—	0.93 ± 0.05	—

A relative amplitude of 1.0 equates to the extent of Ca^{2+} released by 20 μM $InsP_3$; N.D., not determined.

0.3 μM $InsP_3$ in the presence of 50 U/ml and a decrease in the extent of IICR at higher concentrations. A similar biphasic effect on the extent of IICR was also observed with permeabilised SH-SY5Y cells (see amplitude data in Table 1).

Fig. 1B shows the effects of PKA on the extent of IICR from permeabilised RINm5F cells induced by 2 and 20 μM $InsP_3$. Over the PKA concentration range used in this study (up to 400 U/ml) only a stimulatory effect was observed (i.e., a 150% increase in the extent of IICR at 400 U/ml PKA).

In addition, we also investigated the effects of PKA on RBL-2H3 cells which express predominantly the type II isoform of the $InsP_3$ receptor [11]. No effect on the extent of IICR was observed at both maximal or half-maximal concentrations of $InsP_3$ over a range of PKA concentrations up to 400 U/ml (data not shown). Since these cells appeared to rupture during stopped-flow measurements [11], no further work was undertaken with these cells.

Fig. 2 shows the effects of PKA on the transient kinetics of IICR from both cerebellar microsomes (Fig. 2A) and permeabilised RINm5F cells (Fig. 2B). Two concentrations of PKA were investigated in addition to control (i.e., 0, 50, and 200 U/ml), at two concentrations of $InsP_3$, a maximal concentration (20 μM) and an approximately half-maximal concentration (i.e., 0.3 μM for cerebellar microsomes and 2 μM for the RINm5F cells). The lines through the data represent the best fits using exponential rate equations where the kinetic parameters are given in Table 1. The data from the cerebellar microsomes were best fitted to a bi-exponential process as previously shown in [12]. However, the data from the permeabilised RINm5F cells and SH-SY5Y cells had a higher signal-to-noise ratio and therefore could equally well be fitted to a mono- as well as a bi-exponential process. Thus for the sake of simplicity the traces from these samples were fitted assuming a mono-exponential process. For the cerebellar type I isoform at both 0.3 and 20 μM concentrations of $InsP_3$, 50 U/ml PKA stimulated the fast phase rate constant by about 100%. The rate constant for the slow phase was also substantially increased (by about 50% for both 0.3 and 20 μM concentrations of $InsP_3$). This level of increase in the rate constant for IICR was also observed for permeabilised SH-SY5Y cells (Table 1). At an inhibitory concentration of PKA of 200 U/ml, the rate constants reverted back to values close to those observed for controls. Furthermore, the stimulatory effects of PKA (50 U/ml) on the kinetic parameters of IICR could be reversed upon incubation with 85 μg/ml of the PKA inhibitor, PKI. (The rate constant for 20 μM $InsP_3$, in the presence of 50 U/ml PKA, was $1.8\,s^{-1}$, which was reduced back to $1.0\,s^{-1}$ when PKI was also added. Data not shown.) In the case of the type III isoform of the $InsP_3$ receptor, although there was an increase in the extent of Ca^{2+} release with increasing PKA concentration, there was little effect on the rate constants for Ca^{2+} release.

To investigate how PKA affects the channel gating kinetics of the type I isoform of the $InsP_3$ receptor in more detail, single channel recordings were also undertaken on the purified cerebellar $InsP_3$ receptor. Fig. 3A shows portions of the channel recording in the absence

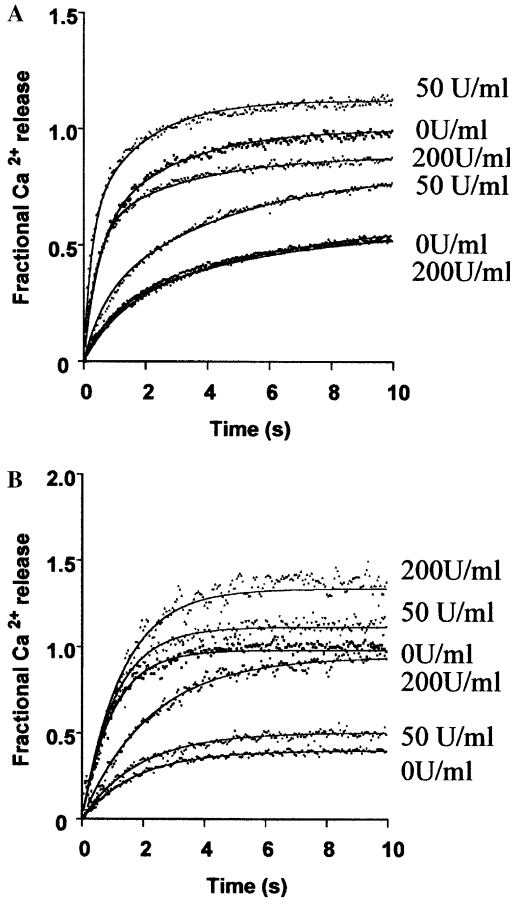

Fig. 2. The effects of PKA on the transient kinetics of IICR from the type I and type III $InsP_3$ receptors. (A) Traces for the time course of IICR from cerebellar microsomes (type I) in the presence of PKA (0, 50, and 200 U/ml) using 20 μM $InsP_3$ (top three traces) and 0.3 μM $InsP_3$ (bottom three traces). (B) Traces for the time course of IICR from permeabilised RINm5F cells (type III) in the presence of PKA (0, 50, and 200 U/ml) using 20 μM $InsP_3$ (top three traces) and 2 μM $InsP_3$ (bottom three traces). The lines fitted through the data are the best fits assuming the kinetic parameters given in Table 1. (A fractional release of 1.0 equates to the extent of Ca^{2+} release with 20 μM $InsP_3$ in the absence of PKA.)

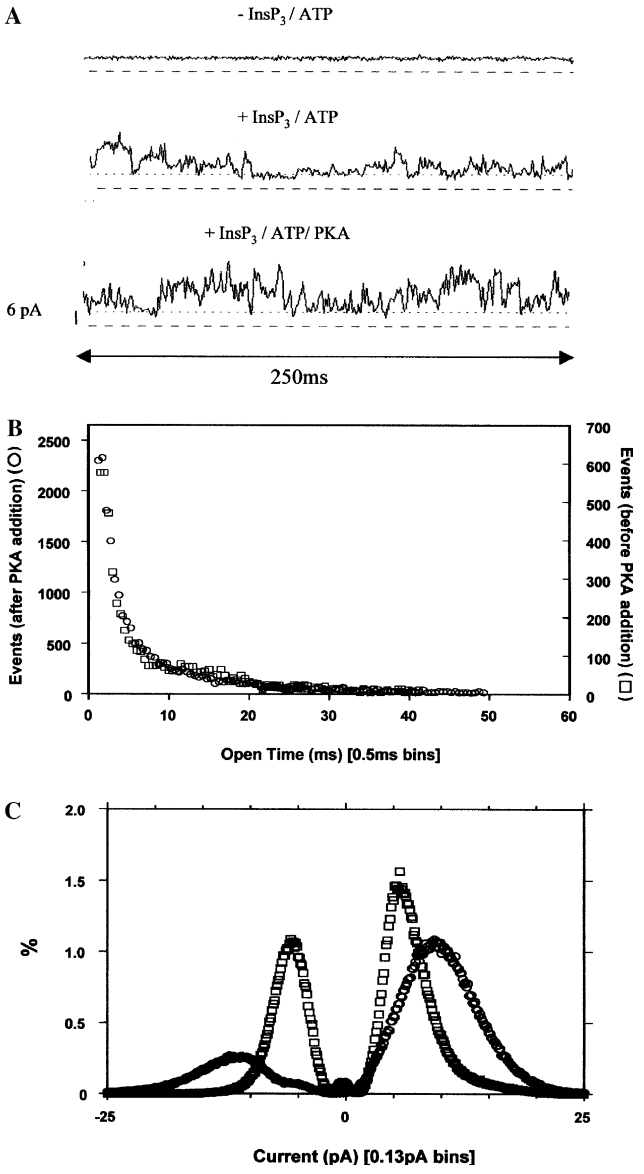

Fig. 3. The effects of PKA on the single channel properties of the type I $InsP_3$ receptor. (A) shows typical 250 ms time sections of channel recordings of the $InsP_3$ receptor in: (top) the absence of ATP (100 μM) and $InsP_3$ (10 μM); (middle) the presence of ATP and $InsP_3$ and (bottom) the presence of ATP, $InsP_3$, and PKA (350 U/ml). The dotted line is the zero current level and the dashed line is at −6 pA. (B) shows a histogram of the number of events versus open time (placed in 0.5 ms bins) before (□) and after (○) PKA treatment. (C) shows the amplitude histogram of percentage time versus current (placed in 0.13 pA bins) before (□) and after (○) the addition of PKA, at an applied potential difference of + and −20 mV.

of $InsP_3$ and ATP, which essentially shows no activity. Upon addition of 100 μM ATP and 10 μM $InsP_3$ (the optimum concentrations for channel activity [15,17]), some activity was observed. This activity is greatly enhanced in the presence of PKA (350 U/ml). The analysis of the properties of the channel, before and after the addition of PKA, is presented in Figs. 3B and C. To assess the effects of PKA on the mean open time of the receptor about 300–400 s of recordings plus or minus PKA were analysed. The number of events of a particular duration (placed in 0.5 ms bins) was plotted as a function of open time (Fig. 3B). The distribution curves of the two data sets could be fitted to a bi-exponential process giving mean open times of 1.8 and 18 ms before PKA treatment and 2.2 and 13.7 ms after PKA treatment. Thus PKA phosphorylation has little effect on the mean open times. The amplitude histogram of the percentage time spent at different conductance levels, before and after PKA treatment from records at both + and −20 mV, is presented in Fig. 3C. From the data the mean channel current was determined to be 6 ± 2 pA (300 pS) before the addition of PKA and this increased nearly twofold to 11 ± 2 pA (540 pS) after PKA treatment. These values were the same at both positive and negative applied voltages, which demonstrates that PKA

phosphorylation does not make the channel voltage-dependent. Thus PKA appears to increase the channel conductance, without affecting the mean open time. This observation contrasts with the results of a recent study, which suggested that PKA did affect the open probability of the $InsP_3$ receptor [22].

Discussion

We have previously suggested that the biphasic kinetic properties of IICR arise from two independent components (i.e., fast and slow components) [11,12]. As these components of biphasic IICR show heterogeneity in their sensitivity to $InsP_3$ [19], and have distinctive pharmacological properties [20,21], we have simply interpreted this as two populations of $InsP_3$-sensitive Ca^{2+} stores receptors which release Ca^{2+} in either a fast or slower fashion. However, it is now clear that when investigating the properties of the $InsP_3$ receptor at the single channel level, they can also adopt different conductance levels. Therefore another degree of complexity may also contribute towards the biphasic process of IICR, especially since the conductance levels can be influenced by external factors such as phosphorylation and modulatory proteins [18]. Therefore a multitude of factors (such as distinct $InsP_3$-sensitive Ca^{2+} pools, variable conductance levels, and extrinsic regulatory proteins) probably contribute towards the manifestation of biphasic IICR transient kinetics.

We have compared the transient kinetics properties of IICR for the $InsP_3$ receptor type I in the presence of PKA with those of the type I receptor using single channel electro-physiological recordings. It is clear that the effect of PKA on the transient kinetics of IICR is complex, with the rate constants and extent of Ca^{2+} release being stimulated at low PKA (up to 50 U/ml), while at higher PKA concentration (200–400 U/ml) these properties decrease. However, when investigating the effects of PKA on the single channel properties of the type I receptor, only a stimulation in channel activity was observed at the 350 U/ml of PKA used. This difference could well be attributed to the experimental conditions used between the two techniques. However, when relating the stimulatory effects of PKA, using both techniques, changes in the rate constants of IICR are unlikely to be due to changes in the mean open time of the channel. There is, however, a good correlation between the increase in conductance levels upon incubation with PKA (i.e., 6–11 pA) with the increase in the rate constants for IICR (i.e., 2.0–4.1 s^{-1}). We cannot at this stage deduce the likely channel gating mechanisms involved in the increase in the extent of IICR which were best observed at sub-maximal concentrations of $InsP_3$, since in the single channel recording experiments, only optimal $InsP_3$ concentrations were used. However, when viewing several seconds of channel recording before and after PKA addition it was noticeable that the periods of little or no activity observed under these conditions in the absence of PKA [15] were not as prominent after channel phosphorylation (see Fig. 3A). These periods of channel inactivity even when $InsP_3$ is bound to the receptor may reflect the bound 'unproductive' state of the receptor that has previously been postulated to exist [11,12]. Therefore, both increased channel conductance and decreased periods of inactivity probably contribute to the increased IICR.

The biphasic effects of PKA on the extent and rate constants of IICR for the type I isoform of the $InsP_3$ receptor also appear to explain the apparently contradictory findings of a number of papers which either show stimulation or inhibition of channel function. Our study shows that either affect can be observed, depending upon the concentration of PKA and conditions used.

Earlier studies [9] showed that there are two serine residues in the type I $InsP_3$ receptor that are directly phosphorylated by PKA (S1589 and S1755). This study also showed that these sites had different potentials for phosphorylation, with S1755 requiring lower concentrations of PKA to become phosphorylated compared to S1589. Therefore the biphasic effects that we observe on the rate constants for IICR could be explained by the fact that, when S1755 is phosphorylated this causes an increase in the rate constants for IICR (presumably by increasing the conductance level), while higher concentrations of PKA are required to phosphorylate S1589, which inhibits the channel activity.

To date, there has been only a limited amount of information on the effects of PKA-dependent phosphorylation for the type III isoform of the $InsP_3$ receptor [10]. From the results presented here we show that the effects of PKA on the type III isoform are distinct from those observed with the type I isoform. Increases in the extent of IICR (without effects on the rate constants) could be due to PKA decreasing the time spent in an occupied unproductive state. Alternatively, it could also be due to a slower process such as channel inactivation dominating the kinetics of the release.

Acknowledgments

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