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Lack of effect on the sodium efflux of the microinjection of D-Ins(1,4,5)P_3 into ouabain-poisoned barnacle muscle-fibers

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A study has been carried out using relatively intact mature muscle fibers from the barnacle *Balanus nubilus* to see whether D-Ins(1,4,5)P_3 stimulates the ouabain-insensitive Na efflux following its microinjection and whether this is accompanied by a contraction of the fiber. Part of the impetus for a study of this type came from the on-going debate between Vergara, Rojas and co-workers and Lea and co-workers, the former group holding the view that skinned barnacle fibers and skeletal fibers in general are responsive to this isomer. The evidence brought forward indicates that the injection of D-Ins(1,4,5)P_3 in concentrations in the range of 10^{-2} M to 10^{-6} M into cannulated *unskinned* fibers pretreated with ouabain fails to increase the residual efflux, and additionally fails to elicit a contraction. A similar picture emerges with the use of non-hydrolyzable DL-Ins(1,4,5)P_3[S]$_3$ analog following its injection in a concentration of 0.5 μM. Higher concentrations of the analog were unavailable for use. This work also involved verification of the idea that an effect might be obtainable in depolarized fibers. However, doubling or tripling K_0^+ and injection of the isomer or the analog simultaneously failed to support this idea. Since nothing is known as to whether D-Ins(1,4,5)P_3 influences the behavior of the Na^+-Ca^{2+} exchanger when operating in the reverse mode, experiments were done to check this possibility. $ATPNa_2$ which is thought to activate Na^+-Ca^{2+} exchange was injected prior to the isomer or the analog but no significant results were obtained. A similar line of reasoning was followed, that of activating the L-type Ca^{2+} channel by injecting $GTPNa_2$ which in addition is known to activate adenylate cyclase. Again, neither the isomer nor the analog were effective. Thus, the only conclusion possible is that in relatively intact, mature barnacle fibers there is no coupling between the phosphoinositide signalling pathway and two other key systems, viz. the Na^+-Ca^{2+} exchanger when operating in the reverse mode and the L-type Ca^{2+} channel. Equally clear is that for some unknown reason the ouabain-insensitive Na efflux and the contractile apparatus are insensitive to D-Ins(1,4,5)P_3[S]$_3$.

Introduction

The object of the present work was four-fold: First, to find out whether the effect of injection of D-Ins(1,4,5)P_3 into ouabain-poisoned fibers mimics Ca^{2+} by increasing the remaining Na efflux and eliciting a contraction. Second, to find out if depolarisation of the fiber membrane with high K_0^+ renders the isomer and its non-hydrolyzable analog DL-Ins(1,4,5)P_3[S]$_3$ more effective. Third, to determine whether or not the isomer or analog influences the behavior of the response of the ouabain-insensitive Na efflux to the injection of $ATPNa_2$ or put differently, the operation of the Na^+-Ca^{2+} exchanger in the reverse mode [1]. And fourth, to answer the question whether the isomer or analog augments the stimulatory response of the Na efflux to the injection of $GTPNa_2$ if the nucleotide does in fact activate L-type Ca^{2+} channels in addition to the adenylate cyclase system (see, for example, Refs. 2 and 3).

The following communication has a direct bearing on the controversy between Vergara, Rojas and co-workers [4,5] and Lea and co-workers [6,7] over the question whether Ins(1,4,5)P_3 acts as a major second messenger for the release of Ca^{2+} from the sarcoplasmic reticulum (SR) in skeletal muscle such as barnacle muscle fibers. Rojas and coworkers, for example, have produced evidence that the injection of Ins(1,4,5)P_3 in a concentration as low as 0.2–8 nM into fibers from the giant barnacle *Megabalanus psittacus* induces strong contractions. However, the application of this isomer by Lea and coworkers to skinned fibers from *Balanus nubilus* fails to induce contracture.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,4,5)P_3[S]$_3$, inositol 1,4,5-tris(phosphorothioate).

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This is also the case with frog muscle. Since this controversy impinges on a related one, namely that the isomer causes the release of Ca^{2+} from isolated SR [8] or that it fails to cause Ca release [9], it cannot be simply ignored.

In order to decide between the first two schools of thought, it seemed well worthwhile to check whether the injection of Ins(1,4,5)P_3 into ouabain-poisoned fibers results in the stimulation of the remaining Na efflux, as well as contracture. The basis of this approach was the rule that the injection of Ca^{2+} in a concentration as low as 1 μM into fibers poisoned with ouabain causes not only a transitory contracture but also a transitory rise in the remaining Na efflux (e.g. Ref. 10). If one assumes that the SR in barnacle muscle fibers possesses IP_3-gated channels and that they behave like SR vesicles reconstituted in planar bilayers, it then follows that the injection of the isomer into ouabain-poisoned fibers should lead to contracture and a rise in the Na efflux, at least in ouabain-poisoned fibers. The alternative to this suggestion is of course the possibility that IP_3-gated channels do not exist in mature barnacle fibers or that they may be of minor rather than major importance vis-a-vis the ryanodine receptor channels, i.e. the Ca^{2+} release channels of the SR.

Materials and Methods

The species of barnacles, the method of dissection, cannulation, microinjection of these fibers and counting of the Na activity in the effluent and the fibers were the same as those described at length by Bittar [10]. The artificial seawater (ASW) used had the following composition (mM): NaCl, 465; KCl, 10; $MgCl_2$, 10; $NaHCO_3$, 10 and pH 7.8. Solutions containing 20 mM and 50 mM K^+ were prepared by reducing NaCl in an osmotically equivalent amount. Solutions for injection were prepared using 3 mM Hepes (pH 7.2). The volume of test solution or 3 mM Hepes solution injected into these fibers was 0.3–0.4 μl. This is supposedly diluted by the myoplasm by a factor of roughly 100. All experiments were carried out at an environmental temperature of 22 to 24°C.

The results given in this paper are expressed as the mean ± standard error. Student's t-test was employed to compare the data statistically. Values for $P < 0.05$ were considered as being significant. Estimates of the size of the observed effects on the Na efflux were calculated on the basis of the rate constant plots. For those few instances where the isomer was injected prior to the onset of peak stimulation by high K^+ the peak effect was taken to be that recorded at the time of injecting the isomer or analog.

All reagents used were analytical grade. Ouabain and Hepes, $ATPNa_2$ and $GTPNa_2$ were supplied by Sigma Chemical Company, St. Louis, Missouri. D- and L-isomers of Ins(1,4,5)P_3 were purchased from LC Services Corporation, Woburn, MA. D,L-Ins(1,4,5)P_3[S]$_3$ was a gift from Dr. Barry V.L. Potter whose present address is School of Pharmacy and Pharmacology, University of Bath, Bath, UK.

Results

Little or no action by injection of D-Ins(1,4,5)P_3

Vergara and coworkers (e.g. Ref. 4), working with chemically skinned muscle fibers of the frog *Rana catesbeiana* were able to show that external application of Ins(1,4,5)P_3 (from Sigma and C. Ballou of the University of California, Berkeley) causes these fibers to shorten. Marked contractions were seen with concentrations of 300 μM. Because electrical stimulation increases the concentrations of inositol phosphate isomers, these workers drew the inference that Ins(1,4,5)P_3 plays a key role in excitation-contraction coupling. However, other workers such as Lea, Griffiths, Tregear and Ashley [6] applied Ins(1,4,5)P_3 to skinned barnacle muscle fibers but saw no contractures. This seemed all the more puzzling since Rojas and co-workers [5] reported that the injection of Ins(1,4,5)P_3 into fibers from the giant South Pacific barnacle *Megabalanus psittacus* elicits contractures with concentrations of the isomer as low as 0.2 μM. These were also seen in the nominal absence of external Ca^{2+}. Blinks, Cai and Lee [11] sought an explanation for these contradictory results and suggested that in skinned frog muscle Ca^{2+} is only released in the presence of Ins(1,4,5)P_3 if the muscle fiber has its T-tubules sealed off, a situation that would allow the passage of current. This comparison seems justified since morphologically speaking invertebrate and vertebrate muscle closely resemble each other: they have the same foot structure with four rounded subunits and a central depression [12].

The results obtained with ouabain-poisoned fibers were as follows: (i) Injection of D-Ins(1,4,5)P_3 in concentrations as high as 10^{-2} M produces a delayed but small and transient increase in the residual efflux (viz. 76 ± 16%, $n = 4$), as illustrated in Fig. 1 (lower panel). Since identical kinetics are obtainable with the L-isomer (viz. 56 ± 10%, $n = 4$) (upper panel), the effect with the D-isomer was dismissed, more particularly since its activity is said to be 2000-times that of the L-isomer [13]. Similar experiments were done using much lower concentrations, e.g. micromolar, but the results were essentially alike. In other experiments, injection of L-Ins(1,4)P_2 in a concentration of 1 μM was also found to be relatively ineffective. Experiments carried out by injection of 1 μM D- or L-Ins(1,4,5)P_3 also show small effects which can be dismissed (Fig. 2).

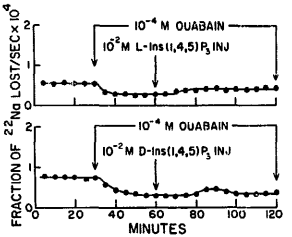

Fig. 1. Upper panel: A negligible, delayed rise in the ouabain-insensitive Na efflux, following the injection of 10^{-2} M L-Ins(1,4,5)P_3. Lower panel: A delayed and slight transitory rise in the ouabain-insensitive Na efflux following the injection of 10^{-2} M D-Ins(1,4,5)P_3 (rate constant plots).

Lack of effect of DL-Ins(1,4,5)P_3[S]$_3$ injection

Strupish, Cooke, Potter, Gigg and Nahorski [13] introduced an analog of Ins(1,4,5)P_3 that is resistant to 5-phosphatase action and only 3-fold weaker than Ins(1,4,5)P_3. The results of experiments show that the injection of 0.5 μM DL-Ins(1,4,5)P_3[S]$_3$ into ouabain-poisoned fibers ($n = 4$) is ineffective. In the representative experiment shown in Fig. 3, it can be seen that there is a slight sustained rise in the residual efflux some 20 min after injection. This type of experiment was repeated more than once: the results were essentially negative. Higher concentrations were not tested because of their unavailability. It is worth mentioning, in this connection, that the EC_{50} for the DL racemate is about 1 μM in studies of Swiss 3T3 cells [13]. And in planar lipid bilayers loaded with heavy SR vesicles up to 40% of the ^{45}Ca is released by DL-Ins(1,4,5)P_3[S]$_3$ in the 2–25 μM range [14].

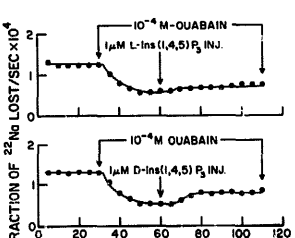

Fig. 2. Upper panel: Kinetics resembling those in the preceding experiment obtained by injecting 1 μM L-Ins(1,4,5)P_3 into a ouabain-poisoned fiber. Lower panel: Slight, sustained stimulation of the ouabain-insensitive Na efflux caused by injecting 1 μM D-Ins(1,4,5)P_3.

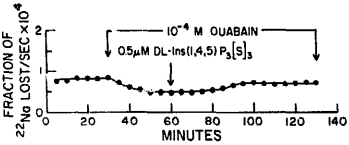

Fig. 3. A delayed but slight and sustained rise in the ouabain-insensitive Na^+ efflux following the injection of 0.5 μM DL-Ins(1,4,5)P_3[S]$_3$.

Elevation of Ko and injection of D-Ins(1,4,5)P_3 and racemic phosphorothioate analog simultaneously

Donaldson, Goldberg, Walseth and Huetteman [15] put forward the suggestion that the voltage across T-tubules may influence Ca^{2+} release from the SR by Ins(1,4,5)P_3. In their experiments with skinned rabbit fibers, they injected the isomer in a concentration of 0.5 μM. When the T-tubules were depolarized, the response was found to be greater than that of polarized tubules. This raised the possibility that a depolarized condition augments the release of Ca^{2+} from the SR following the application of Ins(1,4,5)P_3. In order to verify this possibility, experiments were undertaken with high K_0 and the injection of D-Ins(1,4,5)P_3 or racemic phosphorothioate simultaneously. Justification for the use of 20 mM and 30 mM K_0^+ is to be found in past work, e.g. Mason-Sharp and Bittar [16] who showed that the sudden raising of K_0^+ to such levels causes an increase in the ouabain-insensitive Na efflux, and that the minimal effective concentration is about 20 mM. Under such conditions, the membrane potential is reduced since it is -40 mV, that is 13–16 mV below the resting E_m of cannulated fibers [10]. A resting E_m of -56 mV is still below that of intact, uncannulated fibers since measurements indicate values of -67 mV (e.g. Ref. 17). These considerations are particularly relevant to such experiments since fibers suspended in 10 mM K^+-ASW can be assumed to be in a depolarized condition. The results obtained in these experiments are as follows:

(i) Sudden doubling of K_0^+ and injection of 10^{-2} M D-Ins(1,4,5)P_3 simultaneously shows a prompt rise in the residual Na efflux averaging $85 \pm 12\%$ ($n = 3$), as compared with a value of $110 \pm 4\%$ ($n = 3$) obtained by injecting a 3 mM Hepes solution into companion control fibers and doubling K_0^+ simultaneously. The difference is not significant, P being > 0.1. In parallel experiments K_0^+ was suddenly tripled and 10^{-2} M D-Ins(1,4,5)P_3 injected simultaneously. The results obtained show a stimulatory response of the order of $133 \pm 19\%$ ($n = 3$) vs. $136 \pm 8\%$ in companion controls ($n = 3$). Another type of control experiment was to inject L-Ins(1,4,5)P_3 and simultaneously double K^+. The results of such experiments indicate stimulation in

the ouabain-poisoned fibers of the order of 158 ± 47% ($n = 3$) as compared to a value of 227 ± 18% ($n = 2$) obtained by injection of 3 mM Hepes and doubling K_0^+ simultaneously (P being > 0.2). Notice, however, that these fibers are more sensitive to K_0^+ elevation than the other batches tried – not an uncommon happening. To be more certain that depolarization does not play a role, a separate series of experiments were done in which 10^{-2} M DL-Ins(1,4,5)P_3 was injected and K_0^+ tripled simultaneously. Companion controls were injected with 3 mM Hepes. The results show average responses in each group which are practically the same, viz. 133 ± 19% ($n = 3$) vs. 136 ± 8% ($n = 3$). Taken together, these results mean that Ins(1,4,5)P_3 exerts no effect even when depolarisation of the fiber membrane is increased.

(ii) In the third series of experiments, the effect of 0.5 μM DL-Ins(1,4,5)P_3 injection was tested by doubling K_0^+ simultaneously. The injection of the racemic mixture and doubling K_0^+ simultaneously led to a response the magnitude of which is 158 ± 47% ($n = 3$), as compared with a value of 227 ± 18% ($n = 2$) obtained in companion controls injected with 3 mM Hepes and doubling K_0^+ simultaneously (P being > 0.2). The kinetics of the response in both groups are strikingly alike in that the onset of the response is prompt and reaches a peak within 20–25 min, and subsequently decays rather slowly.

(iii) In the fourth series, 0.5 M DL-Ins(1,4,5)P_3[S]$_3$ was injected and K_0^+ tripled simultaneously, while in companion fibers Ko was tripled and 0.5 M DL-Ins(1,4,5)P_3[S]$_3$ injected close to or after the onset of peak stimulation by 30 mM K_0^+. Representative experiments are given in Fig. 4. The magnitude of the responses is practically the same (516 ± 82%, $n = 5$ in the case of simultaneous treatment and 508 ± 45%, $n = 5$ in the case of successive maneuvers). Here again the values obtained are the same, presumably because the phosphorothioate is without effect.

The fibers used in the preceding experiments were extremely sensitive to high K_0^+, and hence the experiments were repeated using less sensitive fibers. This time, however, the phosphorothioate was only injected simultaneously when K_0^+ was tripled. The results show 112 ± 22% stimulation ($n = 4$) vs. 125 ± 16% in control fibers ($n = 4$). Taken together, then, these results fail to substantiate the notion that reduced voltage amplifies the effectiveness of Ins(1,4,5)P_3 or the phosphorothioate analog.

Lack of effect of isomer and analog after $ATPNa_2$ or $GTPN_2$

The question now arising was: Does the isomer modulate the Na^+-Ca^{2+} exchanger when it is operating in the reverse mode? In order to activate this exchanger, $ATPNa_2$ was injected [1]. This approach was

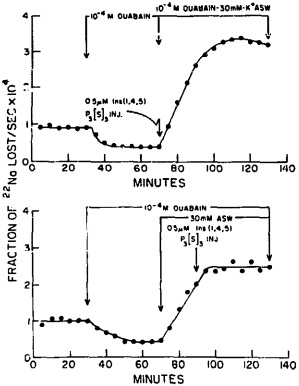

Fig. 4. Upper panel: Stimulation of the ouabain-insensitive Na^+ efflux by injecting 0.5 μM Ins(1,4,5)P_3[S]$_3$ and simultaneously raising external K^+ from 10 mM to 30 mM. Lower panel: Injection of 0.5 μM Ins(1,4,5)P_3[S]$_3$ into a ouabain-poisoned fiber shortly before peak stimulation by a sudden elevation in external K^+ from 10 mM to 30 mM.

employed in view of evidence supporting the hypothesis that $ATPNa_2$ is a positive effector and that Mg^{2+} injection leads to reversal of the stimulatory response to $ATPNa_2$ injection into ouabain-poisoned fibers. Experiments show a lack of effect of injection of 10^{-2} M D-Ins(1,4,5)P_3 ($n = 4$). This was repeated and the results were negative again. These studies were extended to include the injection of 0.5 μM DL-Ins(1,4,5)P_3[S]$_3$. As shown in Fig. 5 (upper panel), neither stimulation nor inhibition occur. Since GTP is known to be a positive effector of the Ca^{2+} channel (L-type) in addition to the adenylate cyclase system (e.g. Refs. 2 and 3), and since the Ca^{2+} channel in barnacle fibers is an L-type channel i.e., DHP-sensitive (e.g. Ref. 18), 10^{-2} M D-Ins(1,4,5)P_3 was injected following the onset of peak stimulation by injecting 0.5 M GTP. As indicated in the lower panel of Fig. 5, there is no effect ($n = 4$). The upper panel confirms this result, namely that the injection of 0.5 M DL-Ins(1,4,5)P_3[S]$_3$ also fails to modify the sustained nature of the response of fibers poisoned with ouabain to the injection of 0.5 M $ATPNa_2$ ($n = 4$). Because of the weight attached to such results, the experiments with $ATPNa_2$ were repeated. But this time only the racemic phosphorothioate analog was injected. Hepes was injected into companion controls. The results of these experiments indicate that the analog had no effect in three of the four test fibers.

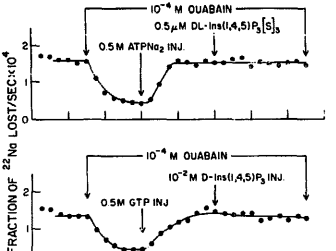

Fig. 5. Upper panel: The lack of effect of injecting 0.5 mM DL-$Ins(1,4,5)P_3[S]_3$ following the onset of peak stimulation of the ouabain-insensitive Na^+ efflux by the injection of 0.5 M $ATPNa_2$. Lower panel: The lack of effect of injecting 10^{-2} M D-$Ins(1,4,5)P_3$ following the onset of peak stimulation of the ouabain-insensitive Na^+ efflux by the injection of 0.5 M $GTPNa_2$.

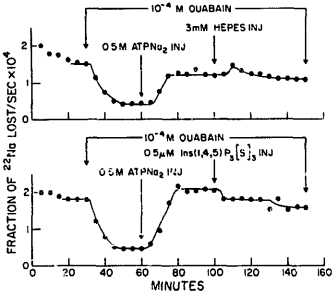

Fig. 6. Upper panel: A control experiment showing the injection of 3 mM Hepes following 0.5 M $ATPNa_2$. Lower panel: Partial reversal by the injection of 0.5 μM $Ins(1,4,5)P_3[S]_3$ of the sustained rise of the ouabain-insensitive Na^+ efflux caused by the injection of 0.5 M $ATPNa_2$.

However, in the fourth a slight reduction in the response was observed. This is shown in Fig. 6 (lower panel). This type of experiment, however, was not repeated.

Discussion

The evidence brought forward here shows quite clearly that the injection of $Ins(1,4,5)P_3$ in low or high concentrations into ouabain-poisoned fibers fails to increase the remaining Na efflux and cause these fibers to shorten. Since as a rule the injection of a 10^{-6} M solution of Ca^{2+} stimulates the ouabain-insensitive Na efflux and this is enough to elicit a contracture [7,10], it is reasonable to conjecture that the sudden introduction into the myoplasm of the isomer fails to release Ca^{2+} from the SR or fails to release enough Ca^{2+}, because barnacle fibers either lack IP_3-gated channels or have a sparsity of them. It is thus possible to posit the idea that if $Ins(1,4,5)P_3$ acts at all, it then elicits considerably less than 1 μM Ca^{2+} release. And if the SR in these fibers represents one-tenth of the total intrafiber volume [19], one is justified on the basis of Ashley's data [20] viz. a myoplasmic free Ca^{2+} of 0.2 μM in the steady state and a free Ca^{2+} of 2 μM following stimulation of these fibers by high K_0^+, to infer that, if anything, $Ins(1,4,5)P_3$ adds little or no Ca^{2+} to the myoplasm. Furthermore, a case could be made that the added isomer is either rapidly phosphorylated by a specific kinase to $Ins(1,3,4,5)P_4$ [21] or slowly degraded. The first is a tenuous argument, of course, in view of the relatively high concentrations of the isomer injected.

The observation of a lack of effect is strengthened by the results obtained with the 5-phosphatase resistant synthetic analog, DL-$Ins(1,4,5)P_3[S]_3$. Though 3-fold weaker than the isomer [13] but not degradable, the observations made are taken to mean that this racemate analog neither increases the ouabain-insensitive Na efflux nor behaves as a messenger of excitation-contraction coupling in these fibers. The fact that only a 0.5 μM concentration was used for injection is not objectionable for at least two reasons. One is that it exerts an effect on Swiss 3T3 cells in the nanomolar range, the EC_{50} being about 1 μM [14]. Second, and more importantly, its regional concentration along the axis of the fiber shortly after release by the micro-injector would be expected to be quite high, e.g., in the high nanomolar range.

The notion that the voltage across the fiber membrane plays a role in the ability of $Ins(1,4,5)P_3$ to release Ca^{2+} is attractive yet untenable according to the data obtained with high K_0^+. This applies to both the isomer and synthetic analog. Further, attempts at clarifying this problem using photolysis have revealed disappointing results, viz. low sensitivity and slow onset of contractions in skinned frog muscle [22]. To make things more confusing, unskinned frog fibers are found by Blinks, Cai and Lee [11] to be irresponsive to the injection of $Ins(1,4,5)P_3$. Ca^{2+} release also fails to occur when 5 or 10 μM $Ins(1,4,5)P_3$ is applied to SR isolated from lobster or rabbit skeletal muscle [23].

But a problem remains. This concerns experiments done with skinned muscle and SR vesicles, especially if the vesicles are placed in planar lipid bilayers (e.g. Ref.

24). Both systems behave in a similar manner in that the Ca^{2+} is free, and the vesicles readily release isotope upon application of the isomer (e.g. Ref. 14). This is the case too when the ryanodine receptor is blocked with ryanodine (e.g. Ref. 24). Thus, the parallelism between skinned (and damaged) fibers and isolated SR vesicles lying in lipid bilayers is striking. The question then immediately to be asked is: Does the bound fraction disappear in both preparations and is it likely that the total free Ca^{2+} in skinned (and damaged) fibers is somewhat larger than that present in the intact fiber? It will be remembered that more than half of the total Ca^{2+} of a skeletal muscle fiber is located in the SR (e.g. Ref. 25a and b), the remaining two large fractions being mitochondrial and nuclear, and that it occurs in the SR in two phases: free and bound. Whether this applies to both the ryanodine-receptor pool and the Ins(1,4,5)P_3-gated pool is not yet known. But in barnacle fibers there seems to be an additional factor which cannot be overlooked. This is that these fibers fall into two classes: those containing about 1 mM Ca^{2+} and those with twice this amount [26,27]. How this influences the distribution of Ca^{2+} in the SR in situ is far from known.

The final point is rather straightforward. It stems from the suggestion that ryanodine-sensitive receptors are plentiful in fetal tissue, e.g. heart muscle, but sparse in mature muscle [28]. One might thus conceive, by analogy, that a similar situation obtains in respect of the Ins(1,4,5)P_3-gated channel and that such channels are sparse in mature muscle. It is then easy to see how the Vergara-Ashley debate could have arisen and that variation in the age, maturity and condition of the fibers investigated may have contributed to the disputed discrepancy. The alternative possibility is that a reduced myoplasmic pCa inhibits the release of Ca^{2+} from the Ins(1,4,5)P_3-gated channel pool [29] especially when the internal free Mg^{2+} is high. However, this is rendered unlikely in view of the observation that the pre-injection of $GTPNa_2$ or $ATPNa_2$ does not enable Ins(1,4,5)P_3 injection to raise the Na efflux.

Acknowledgement

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