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Caffeine-induced Inhibition of Inositol(1,4,5)-Trisphosphate-gated Calcium Channels from Cerebellum

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Effects of the xanthine drug caffeine on inositol (1,4,5)-trisphosphate ($InsP_3$)-gated calcium (Ca) channels from canine cerebellum were studied using single channels incorporated into planar lipid bilayers. Caffeine, used widely as an agonist of ryanodine receptors, inhibited the activity of $InsP_3$-gated Ca channels in a noncooperative fashion with half-inhibition at 1.64 mM caffeine. The frequency of channel openings was decreased more than threefold after addition of 5 mM caffeine; there was only a small effect on mean open time of the channels, and the single channel conductance was unchanged. Increased $InsP_3$ concentration overcame the inhibitory action of caffeine, but caffeine did not reduce specific [^{3}H]$InsP_3$ binding to the receptor. The inhibitory action of caffeine on $InsP_3$ receptors suggests that the action of caffeine on the intracellular Ca pool must be interpreted with caution when both ryanodine receptors and $InsP_3$ receptors are present in the cell.

INTRODUCTION

Inositol 1,4,5-triphosphate ($InsP_3$) is a second messenger used by many cell types to stimulate release of intracellular calcium (Ca) (Berridge and Irvine, 1989) via activation of an $InsP_3$-gated Ca channel (Ehrlich and Watras, 1988; Bezprozvanny *et al.*, 1991; Maeda *et al.*, 1991; Mayrleitner *et al.*, 1991). Another intracellular Ca channel, the ryanodine receptor (Smith *et al.*, 1986; Imagawa *et al.*, 1987; Lai *et al.*, 1988; McPherson and Campbell, 1993), is responsible for Ca release from the $InsP_3$-*in*sensitive Ca pool, presumably via a Ca-induced Ca release mechanism (CICR) (Endo, 1985) or by electromechanical coupling as found in skeletal muscle (Chandler *et al.*, 1976). Recently, both receptor types have been purified to homogeneity (Lai *et al.*, 1988; Supattapone *et al.*, 1988; Anderson *et al.*, 1989; Chadwick *et al.*, 1990; McPherson and Campbell, 1990; Mourey *et al.*, 1990) and cloned (Furuichi *et al.*, 1989; Takeshima *et al.*, 1989; Mignery *et al.*, 1990; Otsu *et al.*, 1990), and some structural similarities between these two channel-forming proteins were found.

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The xanthine drug caffeine has been widely used as an agonist of ryanodine receptor (Sitsapesan and Williams, 1990; McPherson and Campbell, 1993) and as an activator of Ca release from $InsP_3$-*in*sensitive pools (Endo, 1985; Henzi and MacDermott, 1992). Recently, Parker and Ivorra demonstrated an inhibitory action of caffeine on $InsP_3$-induced Ca release in *Xenopus* oocytes (Parker and Ivorra, 1991) that was unlikely to be associated with activation of CICR because the ryanodine receptor is lacking in *Xenopus* oocytes (Parys *et al.*, 1992). An inhibitory action of caffeine on $InsP_3$-induced Ca release from cerebellar microsomes and permeabilized vascular smooth muscle has also been demonstrated (Brown *et al.*, 1992; Hirose *et al.*, 1993), suggesting a direct effect of caffeine on $InsP_3$-gated Ca channels. However, the exact mechanism of the caffeine action on the $InsP_3$ receptor remained to be elucidated.

In the present report we describe the effects of caffeine on the canine cerebellar $InsP_3$ receptor at the single channel level. We found that millimolar caffeine concentrations inhibited the $InsP_3$-gated Ca channels mainly be decreasing the frequency of channel openings and with only a small effect on channel open lifetime. The single channel conductance was not affected. In

agreement with a previous report (Brown *et al.*, 1992), we found that caffeine was not a competitive antagonist of the $InsP_3$ receptor.

MATERIALS AND METHODS

Vesicles

Endoplasmic reticulum (ER) vesicles derived from canine cerebellum were made as described previously (Watras *et al.*, 1991). Briefly, cerebellum (excised from anesthetized dogs) was minced and then homogenized with a Brinkman Polytron (Westbury, NY) in 4 vol of buffer A (5 mM NaN_3/100 μM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA]/20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] pH 7.4). Another 4 vol of buffer A were added to the homogenate, and the suspension was centrifuged for 20 min at 4000 $\times$ g_{max} (Beckman 35 Ti rotor, Fullerton, CA). The supernatant was centrifuged for 30 min at 90 000 $\times$ g_{max} (Beckman 60 Ti rotor). The pellet from the latter spin was resuspended in buffer B (0.6 M KCl/5 mM NaN_3/20 mM $Na_4P_2O_7$/10 mM HEPES pH 7.2) to a final concentration of 4 ml/g cerebellum and centrifuged for 20 min at 4000 $\times$ g_{max} (Beckman 60 Ti rotor). The resulting supernatant fluid was centrifuged for 30 min at 63 000 $\times$ g_{max} (Beckman 60 Ti rotor). The pellet from this last spin was resuspended in buffer C (10% sucrose, 10 mM HEPES pH 7.0) and either frozen in liquid nitrogen (and stored at −80°C) or used immediately for reconstitution experiments.

Bilayers

Vesicles were fused with planar lipid bilayers made from phosphatidylethanolamine and phosphatidylserine (75:25, Avanti Polar Lipids, Birmingham, AL). Bilayers were formed by painting a solution of lipid in decane across a 100-μM hole in a Teflon sheet that bisected a Lucite chamber. The hole was prepainted with a mixture of phosphatidylcholine and phosphatidylserine (75:25) before formation of the membrane. Channel incorporation into the bilayer was followed as described previously (Ehrlich and Watras, 1988) with minor modifications. Briefly, vesicles (~5 μg protein) were added to the *cis* chamber that contained 800 mM KCl, 250 mM HEPES-tris(hydroxymethyl)aminomethane (Tris), and 10 mM $CaCl_2$ pH 7.35. The *trans* chamber contained 53 mM $Ba(OH)_2$, 250 mM HEPES pH 7.35 and was held at virtual ground. Solutions in both chambers were stirred until potassium and/or chloride channels appeared as indicators of fusion of vesicles to the bilayer. Then, the *cis* chamber was perfused with 10 vol of HEPES-Tris solution (250 mM HEPES-Tris pH 7.35, 1 mM EGTA) leaving barium (Ba) on the *trans* side of the bilayer as the only small ion present in the system. Ba was used as the current carrier because the single channel currents are 60% larger and there is a threefold increase in the mean open time of the channels compared to experiments where Ca was the current carrier (Bezprozvanny *et al.*, 1992). With Ba as the charge carrier the $InsP_3$-gated channel spent >90% of the open time in the main subconductance state (see Watras *et al.*, 1991); only openings to this level were considered in the analysis.

Membrane currents were recorded under voltage clamp conditions, and all measurements were made with a transmembrane potential of 0 mV. The *cis* side corresponds to the cytoplasmic side of the ER membrane, and all compounds ($CaCl_2$, $InsP_3$, ATP, caffeine) were added to this side. All experiments investigating the effects of caffeine on the $InsP_3$-gated channel were done using 0.2 μM free Ca on the cytoplasmic side of the channel, which is in the range of free Ca concentrations found to maximize the activity of the $InsP_3$-gated channel (Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991). Because $InsP_3$-gated channels are very sensitive to the changes in the cytoplasmic Ca concentration, the level of contaminated Ca in the stock solution of caffeine (50 mM) used in the experiments was determined by atomic adsorption spectroscopy (Galbraith Labs, Knoxville, TN); the contaminating Ca was measured to be 4.5 μM, too small to cause any significant changes in free Ca in the *cis* chamber that was buffered with 1 mM EGTA. Quantitatively similar effects of caffeine were observed in all experiments performed with three different cerebellar microsomal preparations. Data shown are examples selected from at least four similar experiments unless otherwise stated in the legend. Vesicle fusion occurred in virtually all attempts, but $InsP_3$-gated channels were observed only in 35% of the experiments.

Analysis

Data were stored with a digital tape recorder and analyzed using pClamp (version 5.5 Axon Instruments, Foster City, CA) installed on a personal computer. Data were filtered to 800 Hz by an eight-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized at 2 kHz. Acquired data were filtered further using a digital Gaussian filter (pClamp 5.5) with a cutoff frequency of 400 Hz. Events longer than 2 ms were used for construction of the dwell time histograms and for the construction of amplitude histograms; the threshold used for channel analysis was 2 pA. Evidence for the presence of several functional channels in the bilayer was obtained in some experiments. The number of active channels in the bilayer was estimated as a maximal number of simultaneously active channels during the course of an experiment (Horn, 1991). The open probability for one channel was calculated using the binomial distribution for levels 0, 1, and 2 and assuming that the channels were identical and independent (Colquhoun and Hawkes, 1983).

Where appropriate, values are presented as mean ± SE (number of experiments).

[^{3}H]$InsP_3$ Binding

The effect of caffeine on $InsP_3$ binding was assessed from measurements of specific $InsP_3$ binding to the cerebellar vesicles in the presence of 10 nM [^{3}H]$InsP_3$ and various concentrations of caffeine. Nonspecific binding was measured in the presence of 10 μM nonradioactive $InsP_3$. The procedure used was similar to that described previously (Chadwick *et al.*, 1990). Briefly, 0.05 mg of canine cerebellar vesicles were suspended in a medium containing 50 mM Tris-HCl pH 8.3, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 100 mM NaCl (referred to as standard conditions) in a final volume of 80 μl. In another series of experiments, the vesicles were resuspended in a medium containing 250 mM HEPES-Tris pH 7.35 (referred to as nonstandard conditions). After a 10-min incubation on ice, the samples were centrifuged for 10 min at 12 000 $\times$ g_{max}, and the pellet was washed with the same buffer. After drying, the pellets were solubilized in 0.1 ml Soluine (Packard Instrument, Downers Grove, IL), and the amount of [^{3}H]$InsP_3$ bound to the samples was determined by liquid scintillation counting.

RESULTS

After fusion of canine cerebellar ER vesicles with planar lipid bilayers, channel openings were not observed in the absence of $InsP_3$ (Figure 1A, top trace). Addition of 2 μM $InsP_3$ to the cytoplasmic side of the membrane induced channel openings (Figure 1A, second trace). ATP is not able to activate $InsP_3$-gated channels by itself, but it acts as a potent allosteric activator of these channels (Ehrlich and Watras, 1988; Ferris *et al.*, 1990; Iino, 1991; Bezprozvanny and Ehrlich, 1993). For this reason, 0.5 mM ATP was added before $InsP_3$ to the solution on the cytoplasmic side of the channel. Subsequent addition of millimolar caffeine in the presence of 2 μM $InsP_3$ decreased the probability of finding the channel open (Figure 1A, traces 3–6). Ba was used as a current carrier in this series of experiments, but similar results were obtained with Ca *trans*. An increase in $InsP_3$ concen-

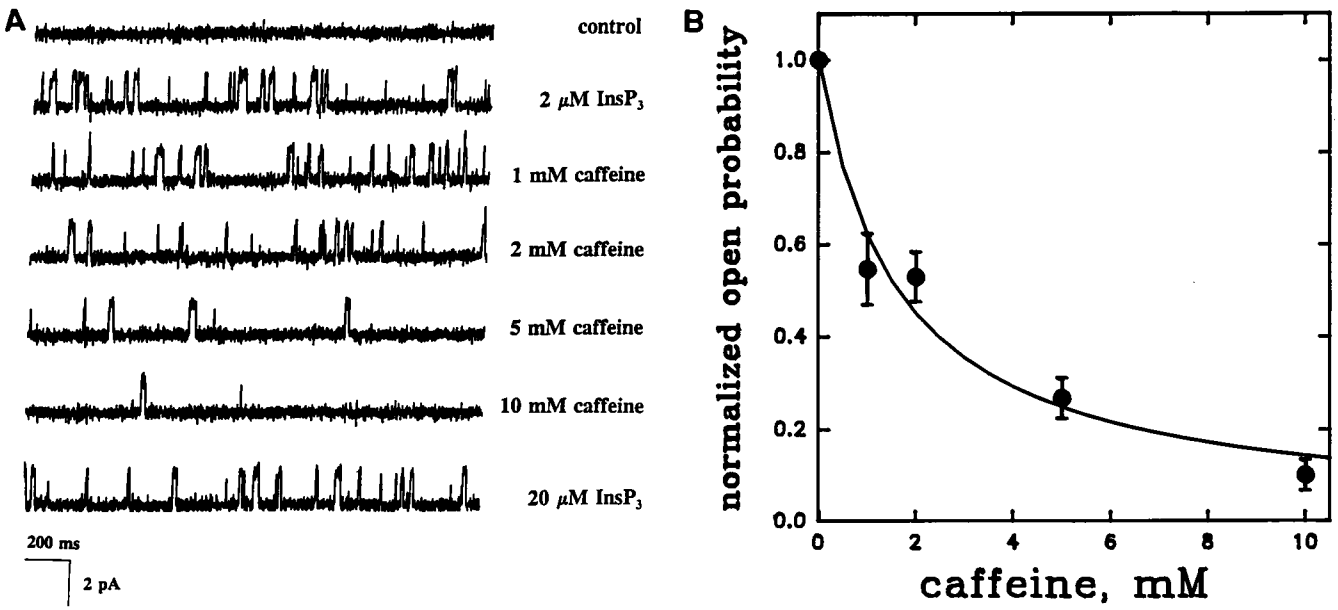

Figure 1. Effect of caffeine on $InsP_3$-gated channels from ER of cerebelum in planar lipid bilayers. (A) In the absence of $InsP_3$, channel activity was not observed (top trace). ATP (0.5 mM) was added to the cytoplasmic side of the channel before $InsP_3$. Addition of $InsP_3$ (2 μM) to the cytoplasmic side of the membrane induced channel openings (second trace) with an open probability of 7.42% for one channel (two channels were estimated to be active in the bilayer in this experiment). Subsequent additions of caffeine to the cytoplasmic side up to concentrations of 1, 2, 5, and 10 mM diminished the open probability of the channel (traces 3–6). The concentration of $InsP_3$ (2 μM) and ATP (0.5 mM) was constant. The bottom trace shows the partial restoration of $InsP_3$-gated channel activity in the presence of 10 mM caffeine after raising the $InsP_3$ concentration to 20 μM. Channel openings indicating divalent cation movement from the *trans* to *cis* side are shown as upward deflections from zero current. Ba is the current carrier. (B) The single channel open probability is plotted as a function of the caffeine concentration on the cytoplasmic side of the membrane. The caffeine concentration ranged between 0 and 10 mM (●, mean ± SE, n = 4). The best fit to the data using Eq. 1 was obtained when K = 1.64 mM. The open probability for one channel was estimated according to the procedure described in MATERIALS AND METHODS under the assumption that all channels active in the bilayer were identical and independent. The open probability of the channels in each experiment was normalized to the single channel open probability obtained in the same experiment before the addition of caffeine. At least 100 s of continuous recording at each concentration of caffeine were analyzed to obtain the open probability.

tration from 2 μM to 20 μM could partially overcome the inhibition of the channels by 10 mM caffeine (Figure 1A, bottom trace). Heparin, a known antagonist of $InsP_3$-gated channels (Ehrlich and Watras, 1988; Supattapone *et al.*, 1988), was still able to completely inhibit the channels even in the presence of 20 μM $InsP_3$ (10 μg/ml of heparin).

The presence of several $InsP_3$-gated channels in the membrane was evident in a majority of the experiments. The single channel open probability at every caffeine concentration used was estimated as described in MATERIALS AND METHODS with the assumption that channels in the membrane were identical and independent. There was some variation in the maximal single channel open probability obtained in different experiments. To compare the data from different experiments, the single channel open probability in each experiment was normalized to the channel open probability obtained in the absence of caffeine for that experiment. The average single channel open probability in the absence of caffeine in this series of experiments was 8.7 ± 0.7% (n = 4). Values obtained after this normalization were used to create the plot of channel activity as a function of increasing caffeine concentration (Figure 1B). The resulting data were fit with the equation:

$$P(x) = \frac{P(0)*K}{K + x} \quad (1)$$

where x corresponds to the caffeine concentration, P is the single channel open probability, P(0) is the channel open probability in the absence of added caffeine, and K is the apparent dissociation constant for the $InsP_3$ receptor-caffeine complex. After the normalization procedure described above, the best fit to the data using Eq. 1 was obtained when K = 1.64 mM (Figure 1B, line). The fit to the data generated by Eq. 1, which implies a Hill coefficient of 1, suggests that the inhibitory action of caffeine on $InsP_3$-gated channels is not cooperative. This result is contrasted with the high degree of cooperativity suggested from the steep concentration dependence observed when these channels are inhibited by high concentrations of ATP and pyrophosphate (Bezprozvanny and Ehrlich, 1993).

To obtain insights into the mechanism of caffeine action, analysis of the single channel behavior was per-

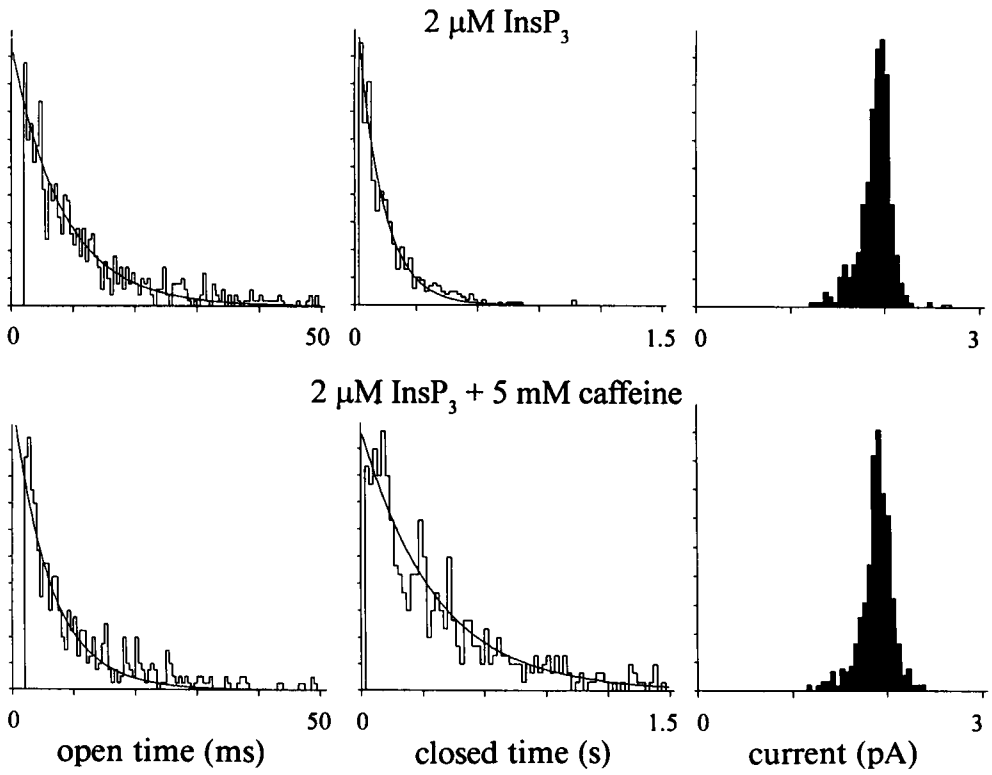

Figure 2. Effect of caffeine on the mean open and closed times and on the single channel conductance of the $InsP_3$-gated channel. Open time distribution (left panels, 0.5 ms/bin), observed closed time distribution (center panels, 20 ms/bin), and current amplitude histogram at 0 mV (right panels, 0.05 pA/bin) are shown without caffeine present (top panels) and in the presence of 5 mM caffeine (bottom panels) for the same $InsP_3$-gated channel. Histograms were constructed from 649 events in the absence of caffeine and 474 events at 5 mM caffeine; all events were ≥2 ms long. Open and closed time distributions were fit with single exponential functions. Openings to subconducting levels of the $InsP_3$-gated channel were infrequent under the conditions used in this series of experiments. The mean current was estimated using a Gaussian fit to the current amplitude histogram.

formed. For the experiment shown on Figure 2 the mean open time of the channels was 8.2 ms in the absence and 6.2 ms in the presence of 5 mM caffeine (Figure 2, left). On average, the mean open time in this series of experiments was 6.3 ± 1.2 ms (n = 4) in the absence and 4.8 ± 0.8 ms (n = 4) in the presence of 5 mM caffeine. Although this difference is significant ($p = 0.049$, using a paired t test), it can account for only 24% decrease in the channel open probability. Thus, caffeine had only a small effect on the mean open time of $InsP_3$-gated channels.

The addition of 5 mM caffeine increased the observed mean closed time of the channels from 120 to 360 ms in the experiment shown in Figure 2 (center). The observed mean closed time of the channels varied considerably from experiment to experiment, most probably reflecting the presence of multiple channels incorporated into the bilayer in different experiments. For this reason, the ratio of the observed mean closed times in the presence and absence of caffeine was calculated in each experiment. On average the addition of 5 mM caffeine produced a 3.3 ± 0.8-fold (n = 4) increase in the observed mean closed time. The increase in the observed mean closed time most likely reflects a decrease in the frequency of channel openings rather than irreversible channel inactivation because the estimated number of active channels in the bilayer was constant at all concentrations of caffeine during the course of each experiment (estimated as described in MATERIALS AND METHODS).

No effect of caffeine on single channel amplitude was observed (Figure 2, right). In this experiment the mean current was equal to 1.94 pA in the absence and 1.92 pA in the presence of 5 mM caffeine, demonstrating that caffeine does not block the ion-conducting pore of the $InsP_3$-gated channel.

As mentioned above, increasing the $InsP_3$ concentration to 20 μM partially restored channel activity even in the presence of 10 mM caffeine (Figure 1B, last trace). On average, the single channel open probability in the presence of 20 μM $InsP_3$ and 10 mM caffeine was equal to 45 ± 17% (n = 3) of the same channel's activity under control conditions (2 μM $InsP_3$ alone). This ability of increased $InsP_3$ concentrations to reactivate the channels in the presence of caffeine seemed to suggest that caffeine acted as a competitive inhibitor of $InsP_3$ receptor. However, when $InsP_3$ binding assays with cerebellar microsomes were performed using standard conditions (see MATERIALS AND METHODS), concentrations of caffeine ≤10 mM had virtually no effect on specific $InsP_3$ binding (Figure 3, open circles), in agreement with previous reports (Brown *et al.*, 1992). When $InsP_3$ binding was measured using the buffer designed for use in single channel experiments (250 mM Tris-HEPES pH 7.35, nonstandard conditions), caffeine had only a weak inhibitory effect on specific $InsP_3$ binding to cerebellar microsomes (Figure 3,

filled circles); the magnitude of this effect (20% inhibition at 10 mM caffeine), however, was insufficient to explain the more than 10-fold decrease in channel activity at the same caffeine concentration (Figure 1B). Therefore, a model more sophisticated than competitive inhibition is required to explain the antagonistic effect of caffeine on $InsP_3$ receptor.

DISCUSSION

In this study we found a direct inhibitory effect of caffeine on $InsP_3$-gated Ca channels from cerebellum at the single channel level. Caffeine inhibited the channels in noncooperative fashion with half-inhibition at 1.64 mM, acting mainly to decrease the frequency of channel openings. Caffeine had no effect on single channel conductance and only a small effect on the mean open time of the channels. An increase in $InsP_3$ concentration in the presence of 10 mM caffeine was able to partially restore channel activity.

The molecular mechanism of caffeine action on the $InsP_3$ receptor could reflect the well-known ability of caffeine to inhibit phosphodiesterase activity (Butcher and Sutherland, 1962). However, the effects of caffeine were observed in an in vitro reconstitution system in the absence of MgATP, making it unlikely that caffeine acts through changes in cyclic nucleotide metabolism. Instead caffeine binding to the $InsP_3$ receptor or a closely associated protein is likely to be necessary for its action. Caffeine in the millimolar range activates the ryanodine receptor. Because the only part of the sequence where $InsP_3$ receptor and ryanodine receptor have significant homology (~40%) (Furuichi *et al.*, 1989; Takeshima *et al.*, 1989; Mignery *et al.*, 1990; Otsu *et al.*, 1990) is their transmembrane domains, caffeine might act at this site on both channels; if this is correct, it is noteworthy that caffeine binding to this hypothetical site has opposite effects on the activity of the ryanodine receptor/Ca release channel and the $InsP_3$-gated channel.

Despite the ability of increased levels of $InsP_3$ to overcome the inhibitory action of caffeine (this report; Parker and Ivorra, 1991; Brown *et al.*, 1992; Hirose *et al.*, 1993), caffeine is not a competitive inhibitor of the $InsP_3$ receptor. In accord with data of Brown and colleagues (Brown *et al.*, 1992), we found that caffeine had no substantial effect on $InsP_3$ binding to the receptor (Figure 3). The same conclusion is supported by indirect evidence. In our previous work (Bezprozvanny and Ehrlich, 1993) we found that inhibition of $InsP_3$-gated channels by high concentrations of ATP and pyrophosphate was highly cooperative. Both of these compounds inhibited $InsP_3$ binding to the receptor (Maeda *et al.*, 1991) and acted as a competitive inhibitor of $InsP_3$-induced Ca release (Iino, 1991) and $InsP_3$-gated channels (Bezprozvanny and Ehrlich, 1993). To fit the steep dose response curve of $InsP_3$-gated channel inhibition by high concentrations of ATP and pyrophosphate a

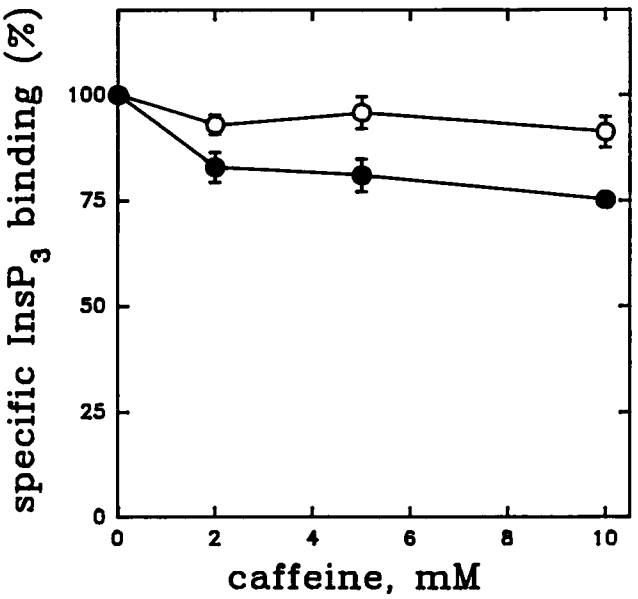

Figure 3. Effect of caffeine on specific [^{3}H]$InsP_3$ binding. Specific [^{3}H]$InsP_3$ binding to the canine cerebellar microsomes was determined in the presence of 10 nM [^{3}H]$InsP_3$ and 0, 2, 5, and 10 mM caffeine. In each series of experiments specific binding was normalized to the value obtained in the absence of caffeine. Specific binding was measured using standard (○, mean ± SE, n = 6) and nonstandard conditions (●, mean ± SE, n = 6); see MATERIALS AND METHODS for details. The average values for specific $InsP_3$ binding in the absence of caffeine were 9.3 ± 0.7 pM/mg (n = 6) for standard conditions and 6.7 ± 0.4 pM/mg (n = 6) for nonstandard conditions.

Hill coefficient of 4 to 5 had to be used (Bezprozvanny and Ehrlich, 1993), whereas no cooperativity in the inhibitory action of caffeine on the $InsP_3$-gated channels was observed (Figure 1B), suggesting that the mechanism of caffeine action is unlikely to result from competition at the $InsP_3$ binding site.

In all the experiments ATP was added at 0.5 mM to potentiate channel openings (Ehrlich and Watras, 1988; Ferris *et al.*, 1990; Iino, 1991; Bezprozvanny and Ehrlich, 1993). Although it is possible that the inhibitory action of caffeine might arise by interfering with the ATP-dependent allosteric activation of the channel, it is unlikely because both mean open and closed times of the channel were altered by ATP (Bezprozvanny and Ehrlich, 1993), whereas caffeine mainly affected the mean closed time of the channel (Figure 2). A direct test of this hypothesis would be difficult because of the extremely low (<1%) open probability of the $InsP_3$-gated channel in the absence of ATP (Bezprozvanny and Ehrlich, 1993).

Insights into the mechanism of caffeine action and ability of raised $InsP_3$ concentration to overcome the effect of caffeine is provided by a simplified model of $InsP_3$ channel gating based on the classical work of del Castillo and Katz (1954) who postulated a model of ligand-gated channel kinetics:

$$\begin{array}{ccccc} & & k_d & \beta & \\ R + InsP_3 & \rightleftharpoons & R\text{-}InsP_3 & \rightleftharpoons & R\text{-}InsP_3^* \\ & & & & \alpha \\ \text{(closed)} & & \text{(closed)} & & \text{(open)} \end{array} \quad (2)$$

where k_d is the affinity of $InsP_3$ binding to the receptor, β and α are the rates of channel openings and closings, respectively. Binding assays have demonstrated (see Figure 3 and Brown *et al.*, 1992) that the k_d is unaffected by the presence of caffeine. Single channel data analysis (Figure 2) revealed that caffeine affects mainly the frequency of openings (the opening rate constant, β), leaving rate of closing (the closing rate constant, α) practically unchanged.

According to the model (Eq. 2), an increase in the $InsP_3$ concentration can only shift the equilibrium between two closed states and does not affect the rate constants α and β. Therefore, to explain an ability of raised $InsP_3$ to overcome the inhibitory action of caffeine (that is, a decreased β), we must conclude that in the presence of 2 μM $InsP_3$ the equilibrium between two closed states is not completely shifted to the right. Thus, one unexpected conclusion is that not all $InsP_3$ binding sites are saturated at 2 μM $InsP_3$ as we previously thought (Watras *et al.*, 1991). This reasoning provides us with a qualitative explanation to the ability of increased levels of $InsP_3$ to recover, at least partially, channel activity (this report) or restore $InsP_3$-induced Ca release (Parker and Ivorra, 1991; Brown *et al.*, 1992; Hirose *et al.*, 1993) in the presence of caffeine. A more complicated model that takes into consideration the tetrameric structure of the $InsP_3$ receptor complex (Supattapone *et al.*, 1988; Chadwick *et al.*, 1990), with an $InsP_3$ binding site on each subunit, must be used for quantitative calculations.

It was previously reported that heparin, a widely used competitive inhibitor of $InsP_3$ binding to its receptor, can act as an activator of ryanodine receptors (Ritov *et al.*, 1985; Bezprozvanny *et al.*, 1993). This action of heparin suggested that caution is needed to interpret the experiments using this compound. The present data, along with previous reports on the action of caffeine (Parker and Ivorra, 1991; Brown *et al.*, 1992; Hirose *et al.*, 1993) indicate that caution is also needed in the interpretation of the effects of caffeine on the intracellular Ca pool if $InsP_3$ receptors are present. This is because the concentrations of caffeine typically used to activate ryanodine receptors (5–10 mM) also decrease the activity of $InsP_3$-gated channels more than 10-fold.

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